

CELL SURFACE GROWTH IN ESCHERICHIA COLI

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Abstract

Autoradiography of various types of cell "ghost" has been used to study the insertion and distribution of certain major outer membrane proteins of the bacterium Escherichia coli. The matrix proteins appear to be inserted at random locations over the entire cell surface and, once inserted, do not appear to be conserved in any fixed spatial location during subsequent growth. A similar indication of random insertion has been obtained for murein lipoprotein. Studies on the major outer membrane protein, protein II* have provided information which suggests that this protein is not evenly distributed over the entire surface of the cell, but is present in concentrated form at the poles. The β lactam antibiotic cephalixin prevents polar concentration of protein II*, but not its incorporation. Upon removal of the antibiotic, protein II* appears to migrate to polar and possibly septal regions. Preliminary studies using electron microscopy support the proposition that protein II* is not evenly distributed over the cell surface.

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Introduction

Growth and division of single cells is fundamental to biological systems, be they bacterial, plant, or animal. The mechanisms involved in eukaryotic cells are, of course, liable to^{be} more complex than those of prokaryotic systems, and ancillary mechanisms involved in cellular differentiation and bulk tissue formation may tend to obscure the basic processes of growth and division. It may well be therefore, that studies on simpler cells, e.g. bacteria, may provide certain basic answers which could be applied to more complex cells. The "simplicity" of bacterial cells is of course relative. Bacterial cell growth and division are highly complex processes in their own right. Escherichia coli has been the organism most widely used in cell cycle studies and is certainly the best understood organism in terms of its molecular biology. It is this organism that has been used in the experimental studies described in this thesis. These studies represent an investigation into the insertion and distribution of certain major outer membrane proteins during cell envelope growth.

Escherichia coli is a Gram-negative organism of regular geometry which can be described as a cylinder with hemispherical ends. Although implicated in nephritis in adult humans and gastro-enteritis in infants, it is generally considered harmless to healthy adults, when contact is made through the usual infective routes i.e. ingestion, inhalation. This factor makes it easy to handle in the laboratory, where it grows well on a wide variety of culture media containing a wide range of carbon sources. Under the best conditions it is capable of achieving a growth rate which results in cell division every twenty minutes.

The genome of E.coli is a single circular DNA duplex (1) about 1200 μ in circumference. Replication of the chromosome is sequential and proceeds from a fixed point, the origin, bidirectionally to another fixed point, the terminus (2). The initiation of chromosome replication has been shown to be dependent on cell size and takes place at a constant cell volume/chromosome origin ratio (3,4). Replication then proceeds at a rate which appears to be constant for growth rates between 20 minutes and 60 minutes per generation. The time taken to complete replication is 40 minutes approximately ('C' time) and this is followed by a delay ('D' time) normally 20' before division occurs (5). Completion of chromosome replication (termination) is a sine qua non for division (6,7) but termination itself is not the trigger that 'signals' division and Jones and Donachie (8) have proposed that division is the culmination of two parallel processes; namely, DNA synthesis which takes 40 minutes, and the synthesis of proteins needed for division (which normally takes place concurrently). In addition, cell division cannot proceed until DNA replication has terminated and there has been a short period of post-termination protein synthesis.

The proposition that the chromosome is attached to the membrane was open to speculation for many years but in recent times a substantial degree of evidence has appeared which supports the attachment theory (9,10), furthermore it has been postulated that the chromosome is attached to the membrane only during replication (9,11). Even more recently, it has been suggested that the chromosome is attached to membrane at its origin of replication and that the membrane component comprising the attachment site is in fact located in the outer

membrane fraction of the cell (12). Whatever the niceties of chromosome attachment are, the simple fact that there is an attachment to membrane implicitly suggests that the way the membrane grows may be an important factor in the segregation of chromosomes during growth and division.

The mode of growth of the cell envelope may also be an important factor in deciding where the division site is located. This site is invariably at the centre of the long axis in normal healthy cells and, as yet, no satisfactory explanation has been produced to account for this regular and precise localisation. Two possible hypotheses can be considered. The first would suggest that division always occurs at the point furthest away from the cell poles (the centre of the cell). As division occurs after a doubling in length, the action of the cell poles in moving apart might control a gradient of chemical activity extending from these poles so that a minimum is reached in the centre of the cell and a septum forms. Difficulties may arise with this hypothesis however in that cell size varies with growth rate (3,13) and therefore cells at different growth rates are different lengths at the time of division. This hypothetical difficulty however, may be negated by the finding of Donachie et al (103) that cells are always the same length twenty minutes before division regardless of growth rate. An alternative hypothesis would involve a few localised zones of growth resulting in areas of old (conserved) and new cell envelope. The division site could be situated at one of these sites or possibly at a junction between old and new areas. Such a mode of growth is found both in other bacteria (e.g. Streptococcus faecalis) and yeasts. vide infra

To determine the importance of the cell envelope in the location of division sites and the segregation of chromosomes, it is essential to determine the mode of growth of the envelope, whether it is a differentiated structure which grows at a few discrete growth sites, or whether it is completely undifferentiated and grows by diffuse intercalation of components at random locations.

2. The Cell Envelope

A. The cell envelope of Escherichia coli consists of three distinct layers, 1) the cytoplasmic membrane, 2) the peptidoglycan layer, 3) the outer membrane (14). The peptidoglycan layer, also referred to as murein or mucopeptide, is located between the inner (cytoplasmic) and outer membranes which are each 75\AA thick and are 100\AA apart (14). This area between the inner and outer membranes is frequently referred to as the periplasmic space though the word space may be a misnomer as it implies emptiness and with an internal osmotic pressure of up to 20 atmospheres (15) in the cell the 'space' must be well filled. In general the Gram negative cell envelope functions as a permeability barrier between the cell and the external medium. It affords protection from harmful agents, e.g. bile salts and detergents, yet allows the uptake of nutrients and permits the efflux of metabolic waste. The envelope is involved in determining the shape of the cell and provides an anchorage for the organs of motility (flagella) and conjugation (sex-pili). The component structures of the envelope will now be considered in more detail.

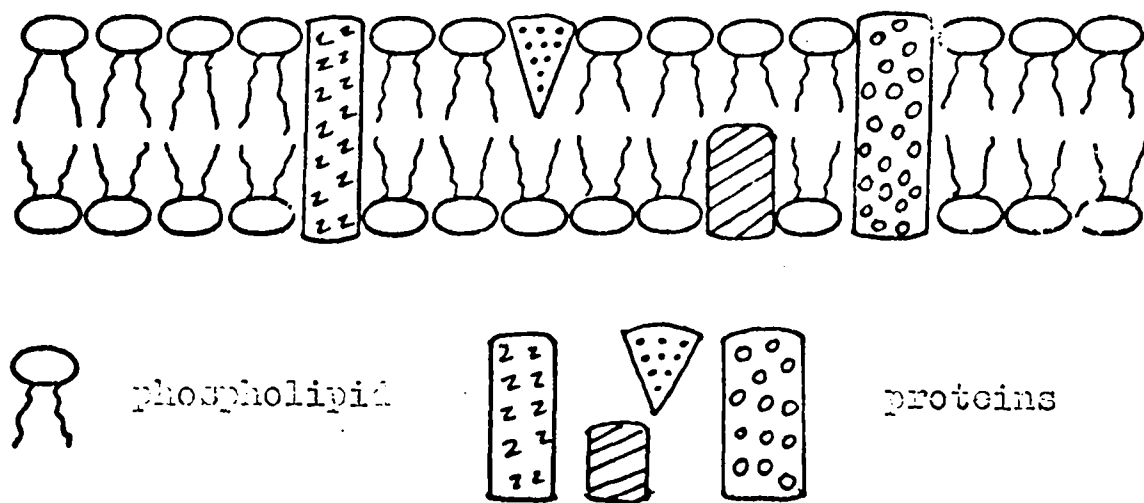
B. The Cytoplasmic Membrane

Efficient separation of inner and outer membranes by techniques introduced by Miura and Mizushima (16) and

subsequently developed by other workers (14,17) have made possible the study of these membranes. The inner membrane is composed of protein and phospholipid (14). The protein comprises about 30% of total envelope protein (18) and the phospholipid consists of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin (14). In brief the composition of this membrane is very similar to that of other biological membranes (19), and the architecture may well be described by the Singer-Nicolson fluid mosaic model (20 and Fig. A). These authors propose that the phospholipid component of biological membranes forms a bilayer, with the hydrophilic polar head groups of the phospholipid molecules facing the outside and the fatty acid chains forming a hydrophobic interior. Protein molecules are postulated to be 'studded' in this matrix, either partially embedded in inner or outer layers or completely traversing the bilayer as in the case of permeases (21). The degree of embedding is thought to be determined by the amphipathic structure adopted by individual protein molecules (20). A possible objection to the idea of a lipid matrix might be that such a structure, being fluid, would exhibit no long range order and thus would provide difficulties in explaining the occurrence of differentiated structures, for example the division septum. It is possible however that 'short range' order might be achieved through protein interactions in specific areas of the membrane surface.

Unlike the outer membrane the cytoplasmic membrane is the site of much enzyme activity. It is in this layer that the basic units of peptidoglycan are assembled (22). Lipopolysaccharide and phospholipid are also synthesised in the cytoplasmic membrane (23).

FIGURE A



Diagrammatic representation of the fluid mosaic model proposed by Singer and Nicolson (20). The phospholipids form a bilayer with the polar head groups facing outwards (circles) and the fatty acid chains facing inwards (wavy lines). Proteins are studied into this bilayer to varying degrees.

C. The Peptidoglycan and Lipoprotein

The peptidoglycan layer of bacterial cells is comprised of a single, giant, bag shaped macromolecule (24), popularly referred to as the "sacculus". The 'backbone' of this structure consists of alternating units of linked N-acetylglucosamine and N-acetylmuramic acid. In the case of Escherichia coli a tetrapeptide of L-alanine, D-alanine, D-glutamic acid and Diaminopimelic acid (DAP) is attached to the muramic acid units and some of these tetrapeptide units are cross-linked to each other - about 50% cross linking in the case of E.coli (24). Although the gross structure of the sacculus has been known for some time (25) it is only recently that details regarding orientation in vivo have been published. Verwer et al (26) have shown that in E.coli the preferential orientation of the glycan chains is perpendicular to the long axis of the cell. This observation would be consistent with the fact that during balanced growth cell diameter is constant (27). Insertion of new glycan chains during growth would lead to elongation but no increase in diameter. It was thought for many years that the peptidoglycan determined the shape of the cell because of its rigidity but although it certainly has shape maintaining properties, this notion is no longer held to be true. Henning et al (28) have shown that murein free 'ghosts' of E.coli can maintain their cylindrical shape and certain halophilic bacteria are known to grow as rods in the complete absence of murein (29). In cell growth studies the peptidoglycan has one extremely useful characteristic; it does not show any marked degree of turnover (29) and once specific labelling has been achieved, utilising the fact that DAP is unique to this layer, no label is lost from it (30).

The peptidoglycan has attached to it a unique protein, the lipoprotein (31). This protein is unique in having at its N-terminus a glycerolcysteine residue to which three fatty acids are covalently bound (32). Approximately 2×10^5 molecules of lipoprotein are covalently bound to the peptidoglycan, the link occurring at every tenth (approx.) peptide unit (31) and the protein extends into the outer membrane. Apart from the bound form of lipoprotein a free form exists in the outer membrane at about twice the concentration of the bound form (33). The combined high number of copies of this protein make it the most abundant protein in E.coli. It has been proposed that the lipoprotein connects the peptidoglycan to the outer membrane, the lipid portion being anchored in the hydrophobic matrix of the outer membrane (34). During the course of this thesis a deletion mutant unable to synthesise either form of lipoprotein was isolated (35). Perhaps surprisingly, this mutant was able to grow and divide normally; it was however hypersensitive to ethylene diamine tetra acetic acid (EDTA), cationic dyes, and detergents, and the authors concluded that the lipoprotein was involved in maintaining the integrity of the outer membrane. This hypothesis is reinforced by even more recent work describing a mutant which lacks lipoprotein and a major outer membrane protein - protein II* (36). Cells lacking both these proteins completely lose their cylindrical shape.

Murein lipoprotein and protein II* are subject to study in the experimental section of this thesis.

D. The Outer Membrane

The outer membrane of E.coli has been subject to intensive study in recent times and much of the information now available

was unknown at the outset of this work.

Although morphologically similar to the cytoplasmic membrane, the outer membrane is functionally very different and contains a unique carbohydrate substance, lipopolysaccharide (LPS) which is only found in the outer membrane of Gram negative bacteria (14). This is also true for lipoprotein (31). Gram positive organisms do not possess an outer membrane. The other components of the outer membrane are phospholipids and protein, the phospholipids being localised almost exclusively in the inner aspect of the proposed outer membrane bilayer (37) and the proteins being relatively few in number of kinds but present in high numbers of copies per cell (14,38). The fact that these proteins are few in number and present in large quantities make them attractive to study in relation to membrane growth, with which this thesis is concerned, and also in the relation to the ways in which molecules can cross biological membranes. There are many investigators in the field of outer membrane proteins at present and almost as many nomenclatures in use to describe these proteins. Table 1 is presented as a comparative guide.

Table 1 Comparison of Outer Membrane Protein Nomenclatures Used by Various Investigators

Henning					Lugtenberg	Mizushima	Rosenbusch	Schnaitman	Mol. Wt.
<i>ompF</i> <i>ompC</i> <i>ompA</i>	Ia	a	0-7	Matrix	3b	40,000			
	Ib	b	0-9		1a	36,500			
	II*	c	0-8		1b	36,000			
	III	d	0-10		3a	33,000			
<i>lpp</i>	IV					17,500			
	(39,40)	(41)	(42)	(43)	(38)	7,500			

Note 1. It is generally agreed that protein IV as designated by Henning is in fact the lipoprotein described by Braun (31).

Note 2 Protein III is regarded by Henning as a major outer membrane protein but does not appear to be so in preparations from other laboratories. McMichael and Ou (44) have suggested that protein III is in fact the monomeric form of type 1 pilin and if this is so it may provide a reason for protein III being more prevalent in Henning's envelope preparations than those of others. Most workers tend to use sonication in the preparation of envelopes, a procedure which would presumably remove most cell pili, Henning does not; he uses a more gently technique first described by Braun et al (45).

Henning's nomenclature will be used in this thesis.

3. Outer Membrane Proteins

a) Proteins Ia and Ib

Proteins Ia and Ib are often referred to as matrix proteins, a term originally coined by Rosenbusch (43), or 'porins' after Nakae (46). They are closely associated with peptidoglycan but not covalently bound to it (40,43). They are involved in the formation of hydrophilic pores, through which solutes with a molecular weight of up to 700 daltons can pass (46,47). Both proteins have been shown to act as receptor protein for bacteriophages: protein Ia for 'phages Tu Ia (48) and T2 (49) and protein Ib for 'phages Tu Ib (48) and 434 (49). The combination of phage receptor activity and association with peptidoglycan have led to the suggestion that these proteins have a 'transmembrane' configuration (48). The relative amounts of proteins Ia and Ib tends to vary with cultural conditions (50), but the combined total number of molecules tends to remain constant at about 10^5 per cell (51). Three genes are known to be involved with proteins Ia and Ib, tolF, meoA and ompB (52 and references therein) and very recent

work suggests that meoA is the structural gene for Ib, tolF is the structural gene for Ia, and ompB is involved in regulation (53,54,55).

b) Protein II*

Protein II* is present in the cell at a concentration of about 10^5 copies per cell (51) and is not associated with peptidoglycan, although it can be cross-linked to it (56). This fact, together with the information that protein II* is the protein part of the receptor for 'phages K3 (57) and Tu II*, (48) has led to the proposition that protein II* has a transmembrane configuration like the matrix proteins (56). Protein II* is "heat modifiable" - a term used to describe the anomalous increase in apparent molecular weight when heated in sodium dodecyl sulphate (SDS) solution (38). This protein is also subject to trypsin digestion and is the only major outer membrane protein to show alteration when whole cell envelope or outer membrane are treated with trypsin (39). A smaller polypeptide is left in the membrane. Evidence has been provided that protein II* plays an important part in F-pilus mediated conjugation (58) and very recently evidence has appeared which suggests that protein II* in conjunction with lipoprotein plays an important role in maintaining cell shape (36). The structural gene for protein II* has been determined and named ompA (59,60).

Table 2 Genetic loci concerned with major outer membrane proteins

Gene*	Synonyms	Map position min (61)	Protein/Function	Reference
ompA	con,tut,tolG	21.5	Structural gene for II*	59,60
ompB		73.8	Regulation of Ia and Ib	54,55
ompC	meoA,par	47.5	Structural gene for Ib	53
ompF	tolF, cmlB	20.8	Probable struct- ural gene for Ia	54,55
lpp	-	36.5	Structural gene for lipoprotein	62

* Symbols to be adopted in the latest revision of the genetic map (63).

c) Recent Research Trends

Much of the recent research in outer membrane proteins has been concerned with how these proteins cross the cytoplasmic membrane and the question of what makes certain proteins outer membrane proteins as opposed to inner membrane proteins. It has now been established that precursor proteins exist for several outer membrane proteins (52) of which the best characterised is probably the precursor for lipoprotein - pro-lipoprotein (64). This precursor protein has an extension sequence of twenty amino acids at its amino terminus and this extension is thought to be of importance in translocating the protein across the cytoplasmic membrane. A model has been proposed (64) for the processing of pro-lipoprotein which suggests that the NH_2 terminal extension acts as a 'signal' to provide a ribosome-membrane junction and subsequent unidirectional crossing of the membrane. The model is similar to the "signal hypothesis" proposed by Blobel and Dobberstein to explain transport of proteins through the cell membrane of

eukaryotes (65). Silhavy et al (66) have demonstrated that β -galactosidase is inserted into the cytoplasmic membrane when the gene for a maltose transport protein (malF) was fused with a lacZ gene (β -galactosidase). It was proposed that a hybrid protein molecule was formed consisting of the N-terminal portion of the maltose transport protein and the C-terminal of β -galactosidase. This result stressed the importance of the part played by the N-terminal in determining protein location.

d) New Membrane Proteins

An intriguing aspect of recent research into outer membrane proteins of E.coli has been the discovery of new major outer membrane proteins. In certain of the mutants which lack outer membrane proteins Ia and Ib a new polypeptide has appeared. This new protein has a molecular weight of around 40,000 and has been variously designated Ic (67) e (68), and E (69). The appearance of the protein does not occur as a direct result of the mutations responsible for the loss of Ia and Ib, but as a result of another mutation at a new locus positioned at 82.7 min on the E.coli linkage map (70). It appears that the new outer membrane protein can function as a pore in the outer membrane and restore at least some of the functions lost when cells lose Ia and Ib (71). The presence of normally silent genes on the E.coli chromosome, which can code for major outer membrane proteins, raises many questions. How many such genes are there? What is the significance of these genes in evolutionary terms? The finding of Chai and Foulds (71) that bacteriophage K3 could use new membrane protein E as part of its receptor as well as another major outer membrane protein - protein II*, has led

these authors to speculate that there may have been a common precursor for major outer membrane proteins. Gene duplication could have occurred followed by mutation in individual genes leading to the production of different major outer membrane proteins.

4. Topology of Membrane Synthesis - A Perspective

Many investigations into the mode of growth of the cell envelope have been carried out over a period exceeding twenty years, and it is now possible to view many of these investigations with the "insight of hindsight". One is still left however with feelings of dismay at the large number of contradictory results which remain as yet unexplained. Some of these studies will now be considered.

Lin et al (72) labelled cells of E.coli with lipid specific compounds (radio-active oleate and glycerol) and allowed them to continue growth in non-radio-active medium. They found no evidence of any sizable areas of conserved membrane. Green and Schaechter (73) reached a similar conclusion after a study on the distribution of labelled lipids after many generations in a mini-cell mutant of E.coli. Experiments involving lipid label in E.coli suffer from the serious drawback that E.coli has two lipid containing structures, an inner and an outer membrane. If these two membranes should grow in different fashions then any segregation pattern in one layer may be masked by the other. Another objection to lipid labelling would be the apparent lateral diffusion of lipid in membranes (74). Morrison and Morowitz (75) used pulse labelling in their experiments with a Gram positive organism - Bacillus megaterium and are therefore not subject to either of the above criticisms. These

authors found that pulsing with tritium labelled palmitate resulted in the preferential labelling of one of the cell poles. In further studies using long term labelling with palmitate the authors concluded that the turnover of incorporated palmitic acid was small and that blocks of membrane were conserved but the labelling procedure employed in this part of the study is open to a serious criticism, in that, the authors used one concentration of palmitate to demonstrate incorporation into phospholipid and another much higher concentration in the labelling experiment. Palmitic acid tends to 'stick' to cell surfaces (unpublished observation) and it is possible that erroneous results could be obtained through non-specific adhesion of the label.

It seems that lipid labelling experiments may be even further complicated by the individual components having varying stabilities (76,77) and that labelling with different compounds may yield different results. Autissier (78) found that labelling lipids with ^3H glycerol gave results which suggested conservation of sizable areas of membrane whilst results obtained with a ^{14}C acetate or ^{14}C oleic acid label suggested random diffusion of phospholipids. Autissier proposed that the glycerol moiety was relatively stable but the phospholipid side chains were subject to rapid turnover. This interpretation however, would be at variance with the conclusion reached by Lin et al (vide supra) and others (79,80). In a very recent study, Pierucci (81) studied the synthesis of phospholipid during the cell cycle of E.coli and found a stepwise change in the rate of synthesis, which occurred around the time of initiation of chromosome replication. The author concluded that her result was consistent

with the concept of zonal growth of the cell envelope at a few discrete sites which double at some time during the cell cycle, in this case at the time of initiation of chromosome rounds.

Stepwise increases in the synthesis of many other cell components have been reported and provide indirect, but not conclusive, support to the idea of zonal growth (82-85). The "indirect" nature of such support must be stressed as there is no real reason to interpret a stepwise increase as being the result of a doubling of growth zones.

Immunofluorescence has been used by Beachey and Cole (86) in an investigation of cell envelope growth in E.coli. Cells were labelled with fluorescent antibody directed against cell envelopes, and grown subsequently in the absence of label. The fluorescent label appeared to be evenly distributed amongst progeny cells with no indication of conservation. It was observed however, that the fluorescent label at cell poles remained, at the original intensity. Although immunofluorescence using whole cell antibody may be of limited value in Gram negative organisms, because of lack of specificity and the presence of extracellular slime (87) there is no doubt that the technique has contributed to the clear, unequivocal evidence in the case of Streptococcus faecalis. This work along with biochemical and microscopical evidence has been reviewed by Higgins and Shockman (88). Cell envelope growth in this organism is completely conservative. The processes of cell enlargement and septation are actually one and the same. New material is added to a central ring which is the site of the next division septum. Autolytic enzymes cleave the new material as it grows, leading to a

situation where two cocci are formed, each consisting of one old and one new hemisphere. Briles and Tomasz (89) have provided evidence for zonal growth in another Gram positive coccus - Diplococcus pneumoniae, the new material being added at distinct equatorial zones.

Autissier and her colleagues have shown in an extremely elegant series of experiments that certain permeases, and by implication the inner membrane in which they are situated, segregate in a few large blocks (78,90,91). These workers utilised the fact that growing cells are much more sensitive to penicillin than dormant cells, thus if a mixture of cells, some of which contain a specific permease and some of which do not, are placed in a medium where the sole carbon source is the sugar that the permease transports, the cells which contain the permease will grow immediately and therefore be susceptible to penicillin induced lysis. The cells which do not contain permease at the outset will not grow until, of course, the sugar induces production of the permease. This gap of a few minutes provided a "window" for Autissier and co-workers to estimate the percentage of a cell population which contained permease at any given time. In a typical experiment cells were pre-induced for a specific permease, e.g. lactose permease, transferred to a non-inducing medium, and at various times returned to a medium containing only lactose as a carbon source and tested immediately for penicillin lysis. It was found that all the cells retained permease until the third mass doubling in minimal medium (fourth in rich medium) when 50% of the cells appeared to have no permease. These observations provided the basis for suggesting a model in which cells growing in minimal medium

have a single, central growth zone and cells growing in rich medium have two growth zones equally spaced along the cell. Other models (as the authors noted) can of course be fitted to the data.

In theory the presence of a unique amino acid (DAP) in the peptidoglycan of Escherichia coli and the fact that turnover or loss of material is negligible (29,30) should have provided ideal conditions for a conclusive labelling study using DAP - auxotrophs. This, however, has not proved to be so: Van Tubergen and Setlow (92) specifically labelled the peptidoglycan of E.coli with ^3H DAP and followed the subsequent distribution of the label amongst progeny cells. They concluded that the peptidoglycan layer grew by intercalating material at random. Lin Hirota and Jacob (72), using a similar technique reached the same conclusion. In later studies however, Ryter et al (30) and Schwarz et al (93) have produced evidence which seems to suggest that new murein is incorporated at the centre of the cell. This result was achieved through pulse labelling with radio-active DAP for short periods (about $1/8$ of a generation). Surprisingly it was found that this new murein was randomised over the whole sacculus within half a generation time. This quick randomisation process would explain the earlier results mentioned above, as these workers used much longer labelling periods, but the mechanisms involved remain a puzzle. In a stable structure like the peptidoglycan apparent randomising of label would have to be achieved via local excision, transfer, and re-insertion. The necessary hydrolytic enzymes to facilitate excision do exist in E.coli but the mechanism for randomisation (and indeed the reason) remains obscure at

present. It is perhaps significant that the authors (93) established a correlation between random insertion and inability to divide in their studies on certain temperature sensitive division mutants. This might suggest that the central insertion zone has more to do with septation than general growth, however they also report that certain other mutants still display central zone labelling when division has been inhibited.

This is probably a suitable point to introduce the possibility of an added complication when carrying out growth studies on rod shaped organisms. Higgins and Schockman (88) and Daneo-Moore and Shockman (94) have proposed that in cocci, surface area increases only by cross wall synthesis, but in rods two different growth mechanisms exist, one for lateral extension and one for cross wall formation. This theory has attracted considerable support and investigators of the action of β lactam antibiotics have proposed that mecillinam inhibits lateral wall extension specifically, leading to coccal forms of E.coli, whilst cephalixin specifically inhibits cross wall formation leading to filamentous growth (95-97).

Iwaya et al (98) have isolated temperature sensitive shape mutants of E.coli which grow as rods at the permissive temperature and spheres at the restrictive temperature. They have correlated their morphological data with information regarding antibiotic sensitivity and shown that the cells were resistant to mecillinam when growing as spheres and sensitive when growing as rods. Another correlation was made with the presence of penicillin binding protein 2 (PBP2) at the permissive temperature (rods) and absence when the temperature was raised (spheres). The authors concluded that

the mutation, rod A (Ts), was in the gene for PBP2 and defects in this gene caused cells to grow as spheres instead of rods. Even more recently Satta et al (99) have used a morphologically conditional mutant of *Klebsiella pneumoniae*, which grows as round cells at pH 7 and normal rods at pH 5.8, to demonstrate an antibiotic sensitivity which varied with prevailing morphology, i.e. sensitive to penicillin G and cephalexin when growing as cocci, resistant when growing as rods. Resistant to mecillinam when growing as cocci, sensitive when growing as rods. These workers also showed a decreased rate of peptidoglycan synthesis when changing from rod to spherical growth and vice versa. They concluded that there were two different sites for peptidoglycan synthesis, one for lateral extension and one for septum formation.

Assuming that two sites for peptidoglycan synthesis exist, problems may arise with labelling experiments through differing rates or modes of synthesis at the two sites, e.g. material incorporated equatorially for a septum may be more concentrated than material incorporated at random locations for lateral extension thus giving the impression that all new material is being incorporated at the centre of the cells.

Models involving conservation and zonal growth of cell membranes are attractive from the point of view of explaining the location of division sites and chromosome segregation. The classic model of this type was proposed by Jacob, Brenner and Cuzin (100) who suggested, as part of their replicon model, that the chromosome was attached to the cell membrane and membrane growth occurred between replicating chromosomes thus providing a mechanism for apportioning chromosomes to daughter cells.

Donachie and Begg (101) developed a model along similar lines based on their observations of individual cells growing on agar coated slides, and the position of potential division sites revealed by the action of a critical concentration of penicillin G. These authors have proposed the "unit cell" hypothesis which suggests that E.coli cells have a minimum length of about 1.7 μm - the unit cell. Each unit cell grows unidirectionally from a single growth site until it reaches twice its original length when a new site is formed. At this time the cell will either divide, giving rise to two cells, each with a single growth site, or if the original unit cell had been placed in a rich medium at the outset so that it would not divide at two unit cell lengths, growth will continue utilising two growth sites. Donachie and Begg originally proposed that the growth site was located at the pole of a unit cell and remained fixed at a distance of 1.7 μm from the other pole during growth. This would predict that after one length doubling the growth site would be in the centre of the cell at which time, if division did not occur, growth would continue between two growth sites of opposite polarity. This model has been modified in the light of further work from these authors, vide infra.

Leal and Marcovich (102) studied the kinetics of appearance of resistance to phage T6 among recombinants after mating a T6 resistant donor with a T6 sensitive recipient. They concluded that the phage receptor sites, which are located in the outer membrane (152), were segregated in large conserved 'blocks'. Begg and Donachie (103,104) have extended this work, utilising an amber tsx mutation in a strain which carried a temperature sensitive suppressor

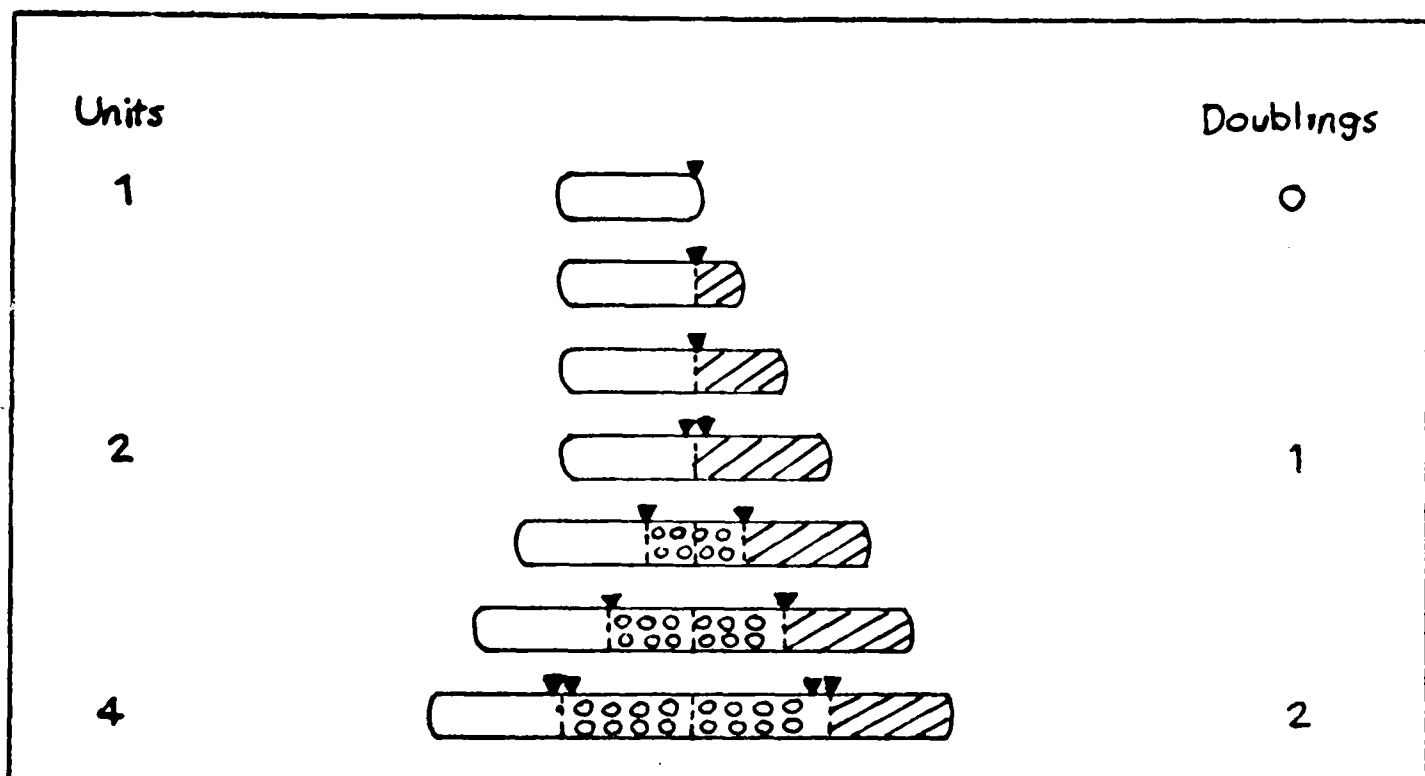
mutation. This system provided conditions whereby cells growing at the permissive temperature had ten times as many receptor sites than when growing at the restrictive temperature. Segregation of T6 receptor sites was observed by labelling the cells with T6 phage, fixing in glutaraldehyde, and observing the distribution of phage under the electron microscope. The authors studied the receptor pattern under a number of conditions and reached the following conclusions 1) T6 receptor sites are incorporated into the outer membrane at random. 2) Once inserted, these receptor sites do not move and are segregated as large blocks. New material was added at one or both cell poles according to the length of the cell. Accordingly the authors have modified their unit-cell hypothesis to take account of the more recent data - see Figures B, C, D.

Ryter et al (105) have studied the integration of the receptor for bacteriophage lambda in the outer membrane of E.coli and concluded that the receptor "is integrated in the envelope during the last quarter of each generation and that the integration process is initiated in the vicinity of the forming septum".

As evidence exists that lipopolysaccharide is inserted over the whole surface (106,107) of the cell it seems possible that not only might individual layers of the cell envelope grow in different fashions, but that individual components of a single layer might do likewise. For this reason models for cell envelope growth obtained by indirect inference from studies on minor envelope components must be viewed with some caution.

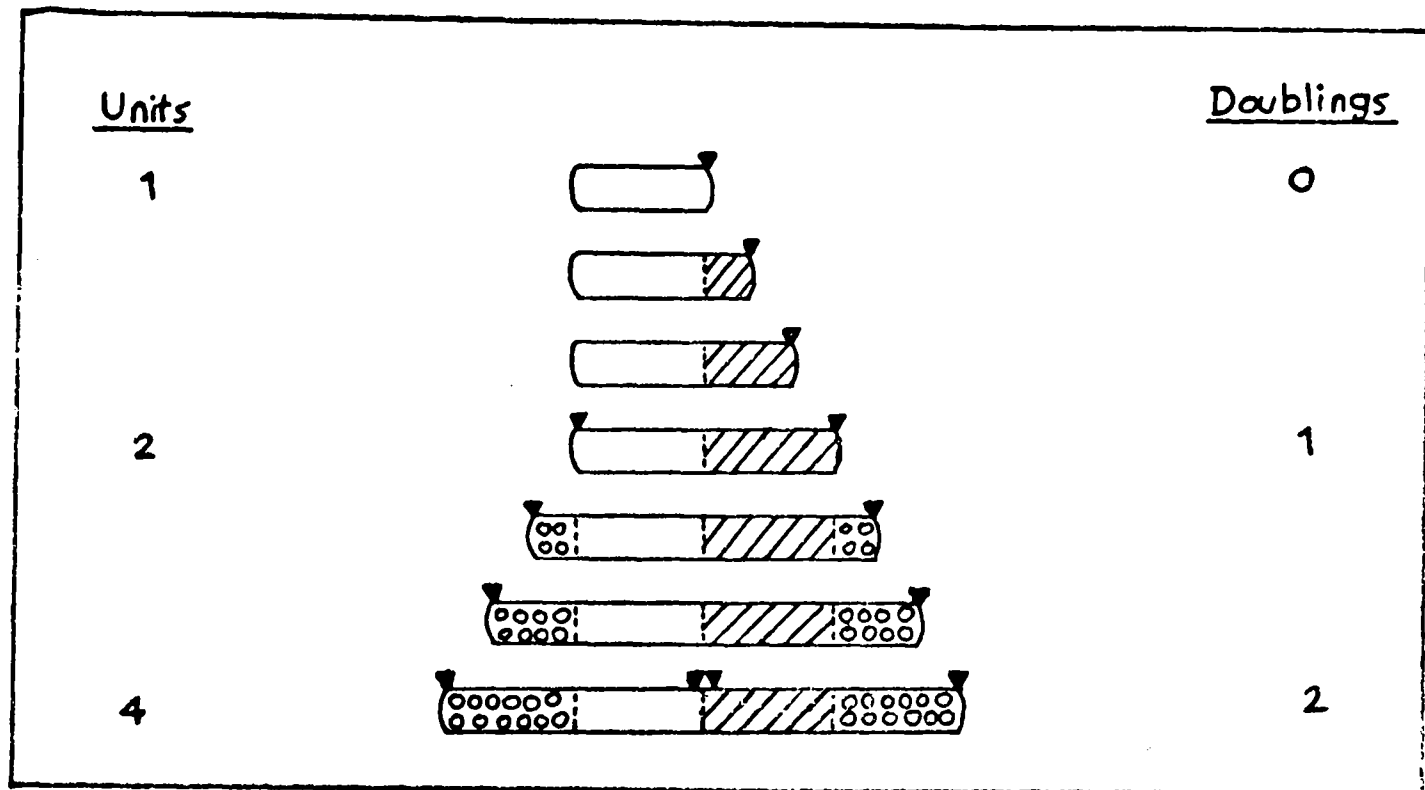
It is apparent that certain basic ground rules must be

FIGURE B



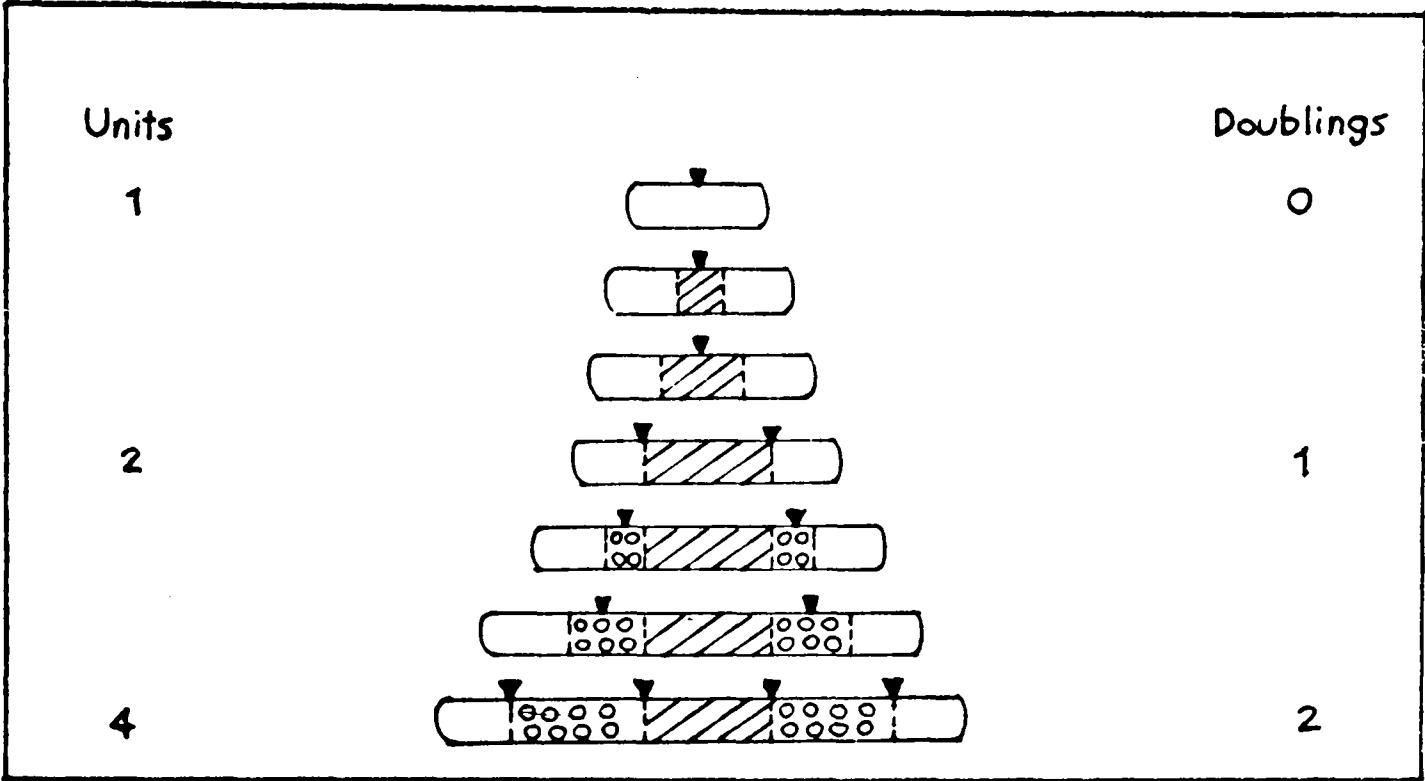
Model for cell envelope growth in *E. coli* based on the unit cell hypothesis of Donachie and Begg (101). The figure shows the progress of a unit cell growing through two generations. (No division is shown during growth eg. cells growing in the presence of low concentrations of penicillin.) The arrows indicate the proposed growth sites. The shaded areas depict growth during each generation. // First 000 Second.

FIGURE C.



Modification of the unit cell hypothesis based on the original data of Donachie and Begg (101) and more recent data obtained from the segregation pattern of phage T6 receptor sites. (103,104). Key as in figure B.

FIGURE D.



Modification of the unit cell model to incorporate central growth zones as proposed by Autissier et al (90).
Key as figure B.

applied when designing labelling experiments in research into cell envelopes growth.

- 1) The labelled component should be specific to one layer of the envelope.
- 2) The labelled component should be a major component of the envelope. (At present the only criterion for determining what is "major" and what is not, is that of quantity.)
- 3) A pulse labelling technique should be applied where possible to avoid possible problems with diffusion or turnover.

Materials and Methods

1. Bacterial Strains

The bacterial strains used are listed in Table 3. Strain P530H was constructed by isolating a spontaneous his⁺ revertant by plating $\sim 10^9$ cells of P530 on plates of minimal medium containing all requirements except histidine. Resistance to bacteriophage T6 was achieved by incubating $\sim 10^8$ cells in the presence of excess T6 phage for thirty minutes and subsequently plating on rich medium for survivors. Strain P530HK3 was obtained by selecting for resistance to bacteriophage K3, (as for T6) checking for resistance to bacteriophage TuII* and screening mutants resistant to both phages by polyacrylamide gel electrophoresis for the absence of protein II*. Strain P530HZ was constructed by cotransducing the temperature sensitive penicillin-binding protein 3 mutation from strain SP63 along with leu⁺.

2. Bacteriophage Strains

Bacteriophages K3 (108) and TuII* (109) were obtained from J. Lutkenhaus and propagated on Escherichia coli P400 by a liquid lysate technique. Cells of P400 in Oxoid nutrient broth No. 2 at a density of $\sim 3 \times 10^8$ cells per ml were infected with phage at a multiplicity of 0.1 and kept in a shaking water bath at 37°C for three hours or until lysis was complete. Chloroform was then added and cellular debris removed by centrifugation. Bacteriophage T6 was obtained from the stocks of this laboratory as was phage P1.

3. Growth Conditions

Cells were grown at 37°C with vigorous rotary shaking (New Brunswick gyratory shaker) in M9 minimal medium (110)

Table 3 List of bacterial strains used

Designation	Genotype/Description	Source/Reference
KM7	K12 F ⁻ <u>thr</u> ⁻ <u>leu</u> ⁻	111
B/r ATCC 12407	Prototroph	112
P400	K12 F ⁻ <u>thi</u> ⁻ <u>argE</u> ⁻ <u>proA</u> ⁻ <u>thr</u> ⁻ <u>leu</u> ⁻ <u>mtl</u> ⁻ <u>xyl</u> ⁻ <u>ara</u> ⁻ <u>galk</u> ⁻ <u>lacY</u> ⁻ <u>rpsL</u> ⁻ <u>supE</u> ⁻ <u>non</u>	113
P530	K12 F ⁻ <u>thi</u> ⁻ <u>argE</u> ⁻ <u>proA</u> ⁻ <u>thr</u> ⁻ <u>leu</u> ⁻ <u>his</u> ⁻ <u>mtl</u> ⁻ <u>xyl</u> ⁻ <u>ara</u> ⁻ <u>galk</u> ⁻ <u>lacY</u> ⁻ <u>ompB</u> ⁻	114
P530H	A <u>his</u> ⁺ <u>tsx</u> ^R variant of P530	This work
P530HK3	a variant of P530H lacking protein II*	This work
SP63	K12 F ⁻ <u>tryE</u> (Am) <u>tyr</u> (Am) <u>ilv</u> ⁻ <u>sup126</u> A cell <u>division</u> mutant <u>temperature</u> sensitive for the production of penicillin binding protein (PBP)3.	B. Spratt and 115
P530HZ	Strain P530H with the temperature sensitive binding protein mutation from SP63 introduced by P1 transduction.	This work

plus glucose 0.2% and required amino acids at 20 µgm/ml or casamino acids at 0.4%. Solid medium for marker testing and strain maintenance was as above plus 1.5% Davis New Zealand agar. Oxoid Nutrient Broth/Agar No. 2 was used for phage lysates and phage sensitivity testing.

Monitoring of Cell Growth

Three parameters were routinely used to monitor bacterial cultures.

- 1) Mass: Mass increase was measured by following optical density at 540 nm using a Hilger spectrophotometer and cuvettes with a 1 cm light path.
- 2) Cell Number: Cell number was estimated by using a Coulter electronic particle counter model ZB fitted with a 30 µm orifice tube. Samples for counting were pre-fixed by mixing with an equal volume of 20% formalin in phosphate buffered saline pH 7.4. This fixation procedure prevents cell shrinkage (116). Subsequent dilution was performed in physiological saline containing 0.1% sodium azide.
- 3) Median Cell Volume: This value was obtained from a Coulter Channelyser attached to the model ZB counter. Median cell volume although different from mean cell volume was a more easily obtainable value and was equally useful.

Bacterial cell cultures were deemed to be in steady state when the increase in cell number paralleled the increase in cell mass and median cell volume was constant.

Buffer Solutions

The following buffer solutions were used for routine manipulations of phage and bacteria -

A) Phage Buffer:

7 g	NaH_2PO_4
3 g	$\text{K H}_2\text{PO}_4$
5 g	NaCl
10 ml	0.1 M MgSO_4
10 ml	0.1 M CaCl_2
1 ml	1.0% Gelatin
1 l	H_2O

B) Phosphate Buffered Saline for bacterial suspension was 0.1 M phosphate buffer pH 7.4 containing 0.4% NaCl.

4. Preparation of Cell Envelopes

40 ml of cell culture at a density of $\sim 10^8$ /ml was centrifuged in an MSE HS18 centrifuge at 7000 rpm for ten minutes at 4°C . The cell pellet was suspended in 5 ml extraction buffer containing 10 mM tris (hydroxymethyl) amino-methane-hydrochloride pH 7.8, 5 mM EDTA (ethylenediamine-tetra acetate) pH 7.8 and 1 mM β -mercaptoethanol. The cells were then ultrasonically disrupted with three twenty second bursts at full power in an MSE 100-W ultrasonic disintegrator. Membranes were then pelleted by centrifuging at 100,000 x G for 45 minutes. The pellet was resuspended in the same buffer by vortexing and again pelleted. The final pellet was suspended in 100 μl of sample buffer containing 62.5 mM tris HCl pH 6.8, 1% SDS (sodium dodecyl sulphate), 5% β -mercaptoethanol, and 10% glycerol.

5. Preparation of Cell Ghosts

a) Rosenbusch Ghosts (43): Cells were washed twice in phosphate buffered saline and suspended in extraction buffer containing 2% SDS, 10 mM tris pH 7.3, 10% glycerol and 0.7 M 2-mercaptoethanol. This suspension was heated to 60°C for thirty minutes. The resulting ghosts were washed three times with distilled water.

b) Cell Sacculi: Sacculi were prepared using the technique

for obtaining Rosenbusch ghosts but using a temperature of 100°C for the extraction.

c) Henning Ghosts: The technique employed was essentially that described by Henning et al (117) but was modified to keep the sacculus intact.

Cells were suspended in 20 mM tris HCl (pH 7.5) containing 40% sucrose and 1% Triton X-100 and left overnight at room temperature. The cells were recovered by centrifuging at 16,000 x G for ten minutes and suspended in 0.5 volume of 4 M Urea. After two hours at room temperature the cells were centrifuged as above, washed twice with 20 mM tris HCl (pH 9.0) and resuspended in 0.25 volume of 20 mM tris HCl (pH 9.0) containing trypsin at a concentration of 1 mg/ml. Trypsin treatment was continued for two hours at room temperature. The resulting cell ghosts were then washed, once with 8 mM magnesium sulphate and twice with distilled water. The lysozyme treatment was omitted from the protocol to leave the mucopeptide intact and thus give more stable rod shaped ghosts.

6. SDS Polyacrylamide Gel Electrophoresis .

Envelopes or ghosts to be analysed were suspended in the sample buffer described in section 4 and heated to 100°C in a boiling water bath for four minutes before being applied to the gel. Electrophoresis was performed according to Laemmli (118) using a Davis Ornstein discontinuous system (119,120).

The separating gel contained 16% acrylamide, 0.094% bis-acrylamide, 0.375 M tris HCl pH 8.8 and 0.1% SDS. This was surmounted by a stacking gel containing 3% acrylamide, 0.08% bis-acrylamide, 0.125 M tris HCl pH 6.8 and 1% SDS.

Both gels contained 0.025% ammonium persulphate and 0.025% TEMED (N, N, N', N'-tetramethylethylenediamine). Both gel mixtures were degassed before the addition of SDS and TEMED.

The slab gel apparatus used has been described (121) and was prepared as follows. The separating gel mix was pipetted in slowly, to avoid bubbles, and overlayed gently with distilled water. When polymerisation was complete (after one hour at room temperature) the water was removed using a syringe and needle and the stacking gel was added. A thirteen well perspex comb was inserted and the stacking gel was allowed to polymerise (again about an hour at room temperature). The comb was removed and the gel clipped to the running apparatus. Running buffer containing 0.025 M tris-glycine pH 8.3, 0.1% SDS and 0.1% β -mercaptoethanol was added to both top and bottom reservoirs and trapped air between the bottom of the gel and the bottom reservoir was removed from between the plates by means of a syringe and bent needle. Protein samples were added to the wells using a micro-syringe and a series of molecular weight standards were included in each gel:

<u>Standard</u>	<u>Molecular Weight</u>
Bovine serum albumin	68,000
ovalbumin	43,000
β -lactoglobulin	18,000
lysozyme	14,000
cytochrome C	11,700

The standards were made up in sample buffer at a concentration of 1 mg/ml and 0.1% bromophenol blue was added as a dye marker.

Gels were run at a constant current of 8 mA for fifteen hours. Samples migrated to the anode.

After electrophoresis the gel was removed from between the glass plates and:

- a) Fixed in 45% methanol containing 9% acetic acid for ten minutes.
- b) Stained in 45% methanol containing 9% acetic acid and 0.1% coomassie brilliant blue for ten minutes.
- c) Destained in 5% methanol containing 7% acetic acid for several hours with four changes of fluid.

All procedures carried out at 37°C on a gentle rotary shaker.

Gels containing 16% acrylamide are extremely stable and easy to handle. When necessary slices were cut out with a sharp razor blade and solubilised in the following scintillation fluid:

720 ml	toluene
80 ml	soluene (Packard Instrument Co Inc, USA)
7.2 ml	1% SDS
2.88 g	PP0 (Packard)
0.081 g	P0P0P (Packard)

Solubilisation took between 24 and 36 hours at 37°C.

Scintillation counting was carried out in a Packard Tri-Carb Scintillation Spectrometer.

7. Autoradiography

High quality glass microscope slides free from scratches and pitting were used (Clay Adams Inc, New York). The slides were boiled in 'Decon' solution (Decon Labs Ltd, Brighton), rinsed well, and stored in absolute alcohol. A gelatin coating was applied to the slides on the day before use by dipping them in Subbing Solution and then drying them in open racks.

Subbing Solution

0.5 g	pure Gelatin
100 ml	distilled water
dissolved by gentle heating then cooled	
0.01 g	Potassium chrome alum.

Stored at 4°C. Warmed to room temperature before use.

Cells were applied to the slides with a wire loop taking care not to scratch the gelatin coating.

Emulsion Preparation

Ilford Nuclear Emulsion L-4 was used and prepared by dissolving 30 g of the emulsion in 20 ml distilled water at 45°C in a dark room using appropriate 'Safe-Light' illumination. The emulsion was stirred gently (to avoid bubbles) for about twenty minutes then transferred to a tube large enough to facilitate dipping slides.

After coating with emulsion the slides were placed in open support racks and dried for four hours in a light-proof box fitted with a spark-proof fan. The slides were then stored in black plastic light-proof boxes until required for developing.

Development of Autoradiograms

Developer and Fixer were made up from standard laboratory reagents according to the Kodak formulary.

D19b Developer

2.2 g	Metol (p-methylamino-phenol sulphate)
72 g	Anhydrous sodium sulphite
8.8 g	Hydroquinone
48 g	Anhydrous sodium carbonate
4 g	Potassium bromide
1000 ml	Distilled water

F24 Fixer

240 g	Sodium thiosulphate
10 g	Anhydrous sodium sulphite
25 g	Sodium metabisulphite
1000 ml	Distilled water

Autoradiograms were developed for 9 minutes, rinsed, fixed for 5 minutes, then rinsed in running water for ten minutes.

Slides were stained with a 1% solution of Methyl Violet (pre-filtered through Whatman No. 1 filter paper), mounted in

water under a Chance No. 1 coverslip, and examined using a Zeiss photo-microscope.

8. Radio-active Labelling Procedures

All radiochemicals were obtained from The Radiochemical Centre, Amersham, England.

L- 2,5 ^3H Histidine had a specific activity of 43 Curies per mMol.

L- 4,5 ^3H Leucine had a specific activity of 51 Curies per mMol.

Pulse Labelling

Growing cells were concentrated by filtering the culture through a 47 mm diameter Millipore assembly (Millipore Corporation) fitted with a 0.45 μm pore size membrane and resuspending in a small volume of growth medium containing ^3H histidine at a concentration of 20 μ Curies per ml for the required pulse time. The pulse was stopped by washing the cells on a Millipore membrane with phosphate buffered saline pH 7.4 containing non-radio-active histidine at 100 $\mu\text{g/ml}$. Pulse labelling with ^3H leucine was performed in similar fashion.

9. Antibiotics

Penicillin G was obtained from Dista Laboratories and used at either 30 I.U/ml or 60 I.U/ml to inhibit cell division but not growth (122).

Cephalexin was a kind gift from Eli Lilly and Co and was used at a concentration of 40 $\mu\text{g/ml}$ to inhibit division (123) but not growth.

Nalidixic acid was obtained from Calbiochem and was used at a concentration of 20 $\mu\text{g/ml}$ to inhibit division but not growth.

10. Transduction using P1 Phage

P1 transducing lysates were prepared by a plate lysis technique (124). 5 mM Calcium chloride was present at all stages. Recipient cells were grown overnight in nutrient broth medium. 0.2 ml overnight culture was inoculated into 1 ml of nutrient broth containing 0.05 ml of 0.05 M calcium chloride and approximately 5×10^7 transducing phage were added. After 15 minutes incubation at 37°C the cells were centrifuged and resuspended in 0.1 M sodium citrate solution pH 7.0. 0.2 volumes were spread on the appropriate selective medium and plates were incubated at the required temperature (30°C when transducing a temperature sensitive marker) until transductant clones appeared. Individual clones were then streaked out again on selective medium and subsequently tested for all relevant markers.

11. Electron Microscopy

K3 Labelling Technique

Cells were grown to a density of $\sim 2 \times 10^8$ per ml in Oxoid Nutrient Broth No. 2. Two volumes of culture were mixed with one volume of K3 phage lysate ($\sim 8 \times 10^{10}$ pfu/ml) to give a phage multiplicity of 200 per bacterium. Adsorption was stopped by adding three volumes of glutaraldehyde fixative.

Fixative

5 ml Glutaraldehyde (TAAB Laboratories, Reading, England)
20 ml 0.1 M Phosphate buffered saline pH 7.4

Cells were pelleted and resuspended in glutaraldehyde fixative for one hour. This procedure was followed by washing three times in distilled water.

Grid Preparation

Equal volumes of cell suspension and an aqueous solution

of bacitracin 40 µg/ml were mixed and applied to Collodion coated copper grids. The bacitracin facilitated even spreading (125) of the uranyl acetate stain which was then applied at a concentration of 1.5%.

All solutions used in procedures for electron microscopy were filtered through a Millipore membrane pore size 0.22 µm before use.

Grids were examined in a Siemens Elmiskop 1A electron microscope.

12. Collection and Treatment of Data from Autoradiograms and Electron Micrographs

Autoradiograms were examined under phase contrast in a Zeiss Photomicroscope. The unusual combination of examining stained preparations with phase-contrast optics was found to be extremely effective in the case of cell ghosts which were too 'ethereal' to be visualised under bright ground conditions after staining, and were equally invisible when examined with phase contrast before staining. Photographs were taken on 35 mm film (Ilford PAN-F ASA50), chosen for its fine grain characteristics, at a magnification of x400 to the negative. The film was processed using Ilford ID11 developer and Ilford Hypam fixer. Prints were then made on Ilfobrom high contrast paper at a further magnification of x4. Ilford PQ Universal developer was used in the printing process.

Grain position was measured using a metric ruler and expressed as the fractional distance from the nearer of the two cell poles.

Electron Micrographs were prepared using Ilford SP353 photographic plates and processed with Ilford PQ Universal developer

and Ilford Hypam fixer. The magnification was $\times 16,700$ to the negative. Prints were made at a further magnification of $\times 3$ on Ilfobrom soft grade paper. Phage positions were measured and expressed in the same way as grains from the autoradiograms.

Introduction to Experimental Studies Section

A. Rosenbusch (43) has described a technique which produces rod-shaped cell "ghosts" from E.coli which consist of the cell sacculus surrounded by a major outer membrane protein, which he termed matrix protein. Lugtenberg et al (50) have since shown that "matrix protein" isolated from B strains of E.coli (Rosenbusch used a B strain) consists of a single protein (Ia) but comprises two distinct proteins when obtained from K-12 strains (Ia and Ib). The bound form of murein lipoprotein (31) is also present in the ghosts.

This suggested that such ghosts could be used to determine the localisation of matrix protein during cell growth. Murein lipoprotein contains no histidine (31) so the use of ^3H histidine ensures specific labelling of the matrix protein/proteins which comprise approximately 35% of the total outer membrane proteins (50). Cells of E.coli K-12 and B/r were pulse labelled with ^3H histidine and subsequently allowed to continue growing in the presence of low concentrations of penicillin to inhibit division. Experiments involving pulse labelling of pre-formed filaments are also described.

B. When Rosenbusch ghosts are heated in the presence of SDS to temperatures above 60°C , the matrix proteins are removed from the peptidoglycan (43) leaving murein lipoprotein as the only protein on the sacculus. Labelling cells with ^3H leucine and then preparing sacculi in this way has therefore allowed a study of the localisation of the bound form of murein lipoprotein.

C. Henning et al (28) have described a technique for obtaining cell ghosts from E.coli which largely consist of four major outer membrane proteins I (Ia + Ib), II (derived from the action of trypsin on II*), III (44,151) and IV (Braun's lipoprotein). This technique has been slightly modified - see Materials and Methods - to facilitate the production of ghosts from strains which lack either proteins Ia and Ib, or Ia, Ib and II*. The use of tritiated histidine has permitted the preparation of cell ghosts which contain only one or two labelled major outer membrane proteins. Labelling studies on these mutants under a variety of conditions are described in section C. Preliminary findings from a study using electron microscopy as an alternative to autoradiography are also presented.

Experimental Studies

A. An investigation into the insertion and distribution of the matrix proteins of *Escherichia coli*

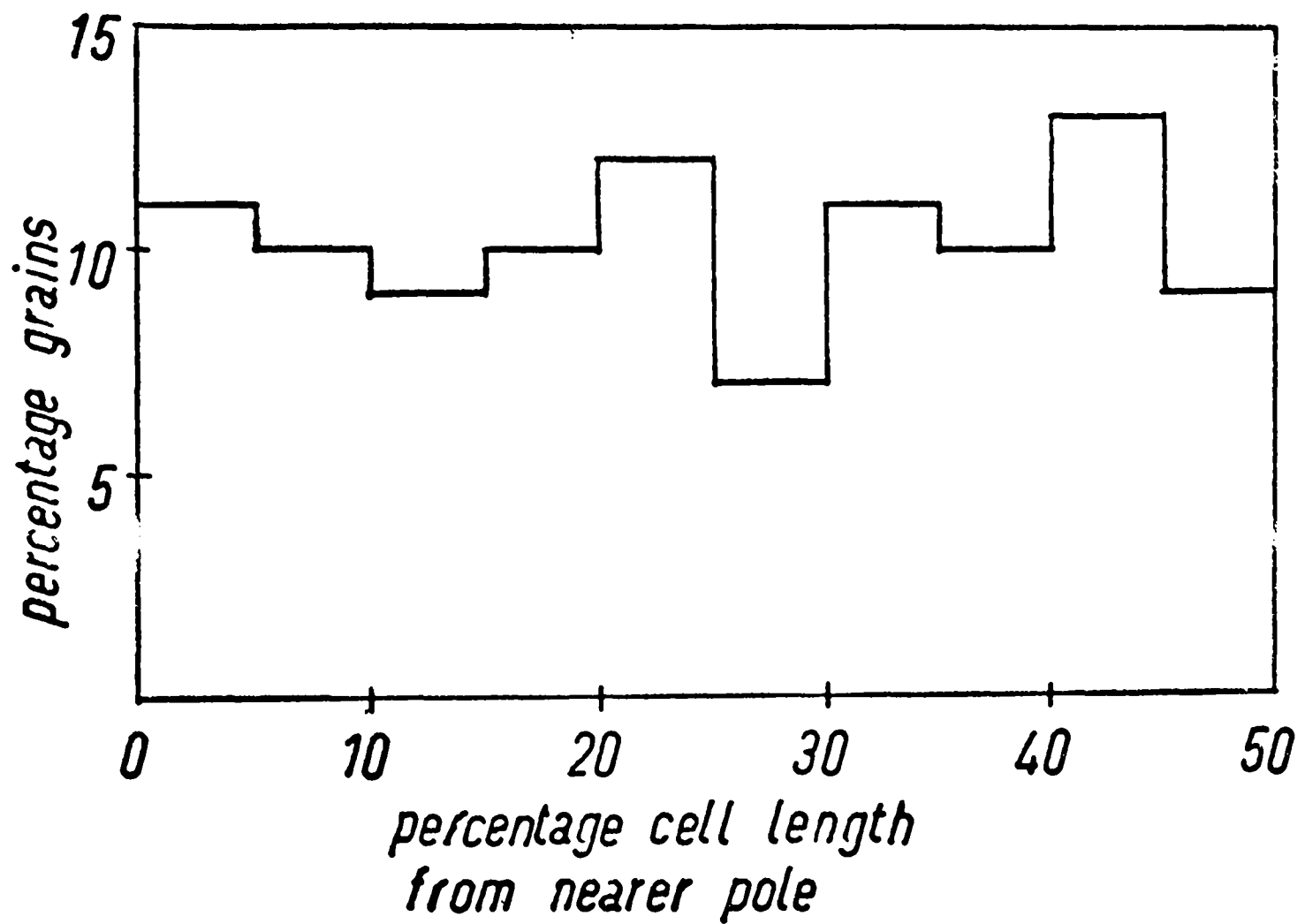
Cells of *E.coli* K-12 KM7 were pulse labelled with ^3H histidine for 10 min, chased with unlabelled histidine and allowed to continue growing for two mass doublings in the presence of penicillin G (60 IU/ml). Autoradiograms of Rosenbusch ghosts made from these cells were prepared and grain positions measured. (All techniques were as described in "Materials and Methods".) The penicillin prevented division without affecting the cell growth rate in mass or length (101,103) and thus made interpretation of grain pattern easier. Strain KM7 was chosen as a K-12 strain which can easily achieve two mass doublings in the presence of penicillin.

Figures 1 and 2 show that grains were randomly distributed over the entire surface of the ghosts.

Other workers have shown that the matrix proteins are the sole proteins present (apart from the bound form of murein lipoprotein) in Rosenbusch ghosts (43,50). This was confirmed for the KM7 ghosts from the above experiment, by means of polyacrylamide gel electrophoresis (PAGE). The bound form of murein lipoprotein remains bound to the cell sacculus after heating to 100°C in SDS and therefore does not enter the gel. The lipoprotein can be released by disrupting the sacculi with lysozyme but is difficult to visualise in PAGE systems because of its low molecular weight (~ 7500).

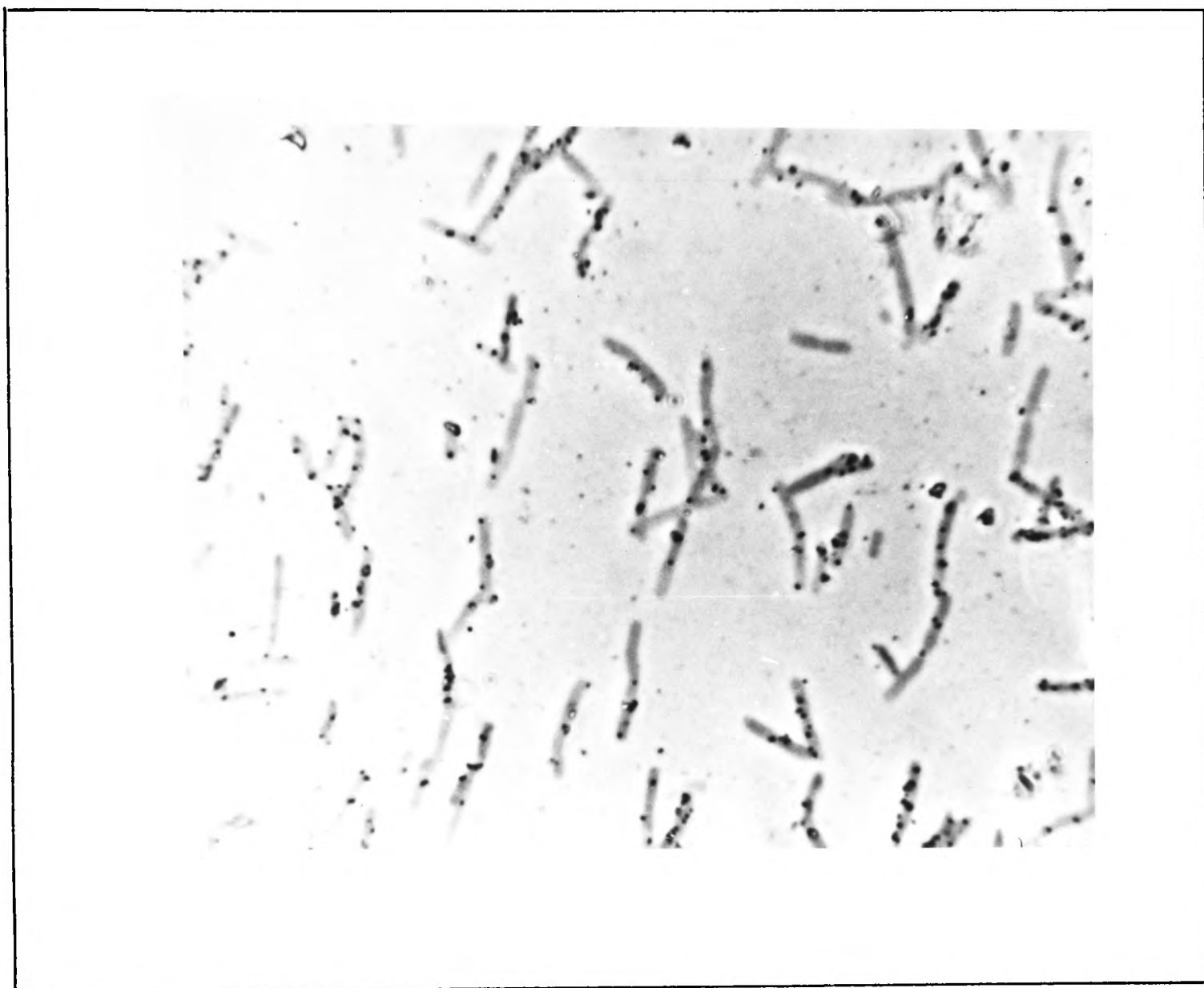
To ensure that ^3H histidine was not being incorporated into the cell sacculus, the murein lipoprotein, or any previously undetected low molecular weight protein, a control

FIGURE 1



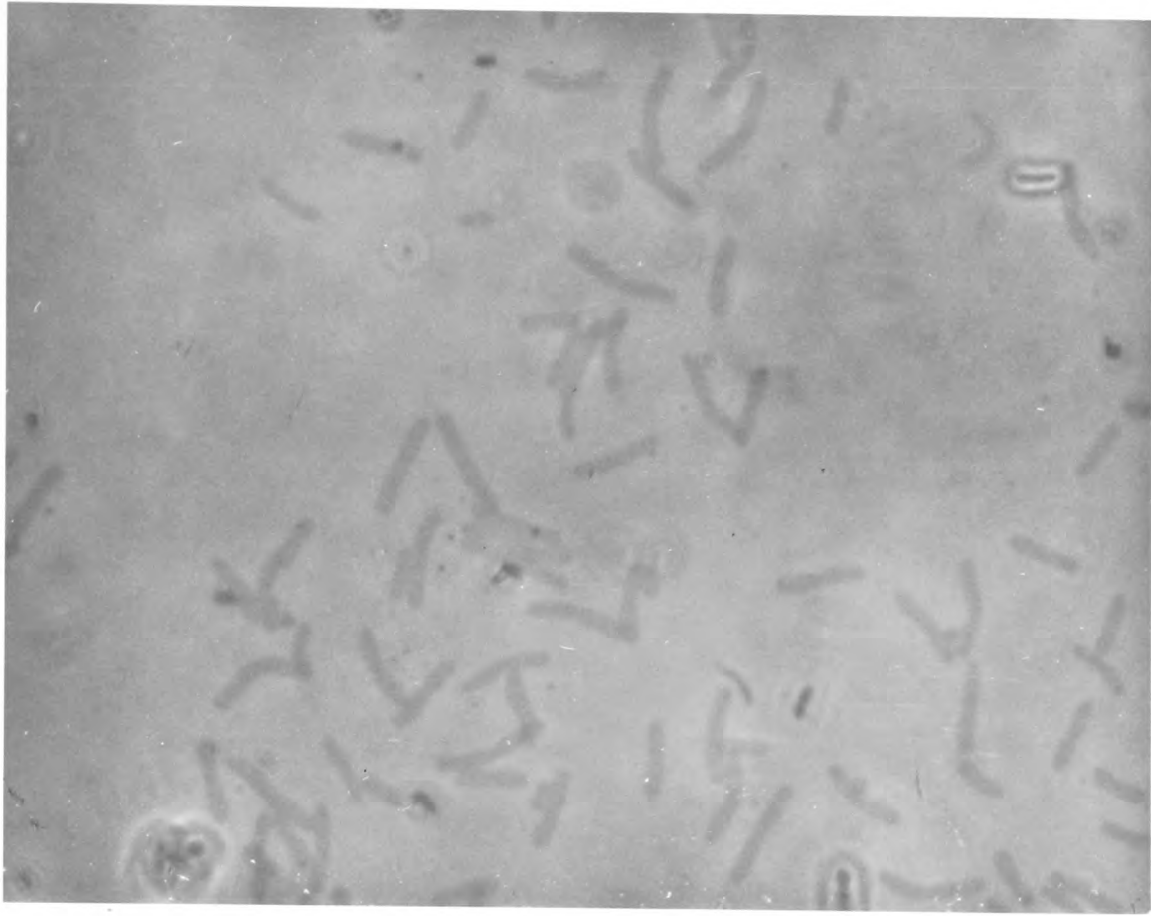
Distribution of grains (304 grains measured) on the surface of cell "ghosts" of E.coli. KM7. Cells were pulse labelled with (3H) histidine for 10 min. and subsequently grown for two mass doublings in the presence of penicillin 60 I.U./ml. The distance of each grain from the nearer pole was measured and expressed as a fraction of the total cell length.

FIGURE 2.



Photomicrograph of "ghosts" described in Figure 1. X 1500.

FIGURE 3.



Photomicrograph of sacculi prepared from ^3H histidine labelled ghosts of *E.coli* KM7 cells by heating to 100°C in S.D.S. to remove matrix protein. X 1500.

experiment was performed. Rosenbusch ghosts from the above KM7 experiment were heated to 100°C to remove the matrix proteins. Autoradiograms were prepared from the resulting sacculi and exposed for the same period of time as the experimental preparations. Figure 3 shows that the sacculi are unlabelled.

Figure 4 shows the grain distribution obtained when cells of E.coli B/r were pulse-labelled with ^3H histidine for 5 min and subsequently treated in similar fashion to the KM7 cells in the previous experiment. E.coli B/r contains only one species of matrix protein - Ia (50) so the grain distribution represents the distribution of a single major outer membrane protein which is present at a concentration of about 10^5 copies per cell (43).

The grain distribution once again is clearly random.

The results shown above suggest that matrix protein/ proteins synthesised during the period of a short pulse of ^3H histidine are distributed over the entire surface of the cells some two generations later.

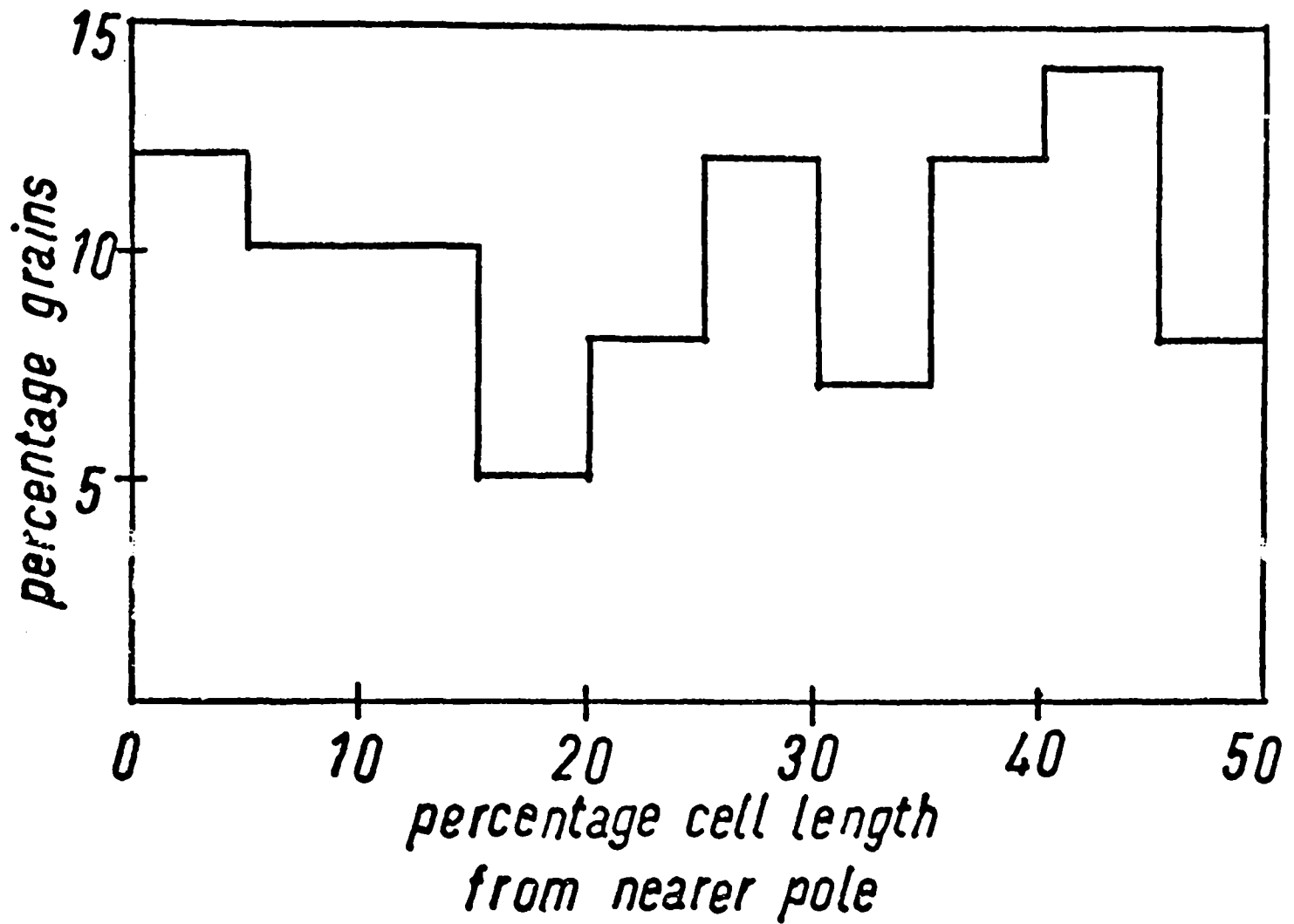
The possibility of localised insertion of matrix protein or short term localisation was now investigated.

Filaments of KM7 cells were formed by treating growing cells with penicillin G (60 IU/ml) for two mass doublings. The filaments were pulsed with ^3H histidine for 1 min immediately before ghost preparation and autoradiograms prepared.

Figure 5 shows that the grain distribution over the cell surface is again random.

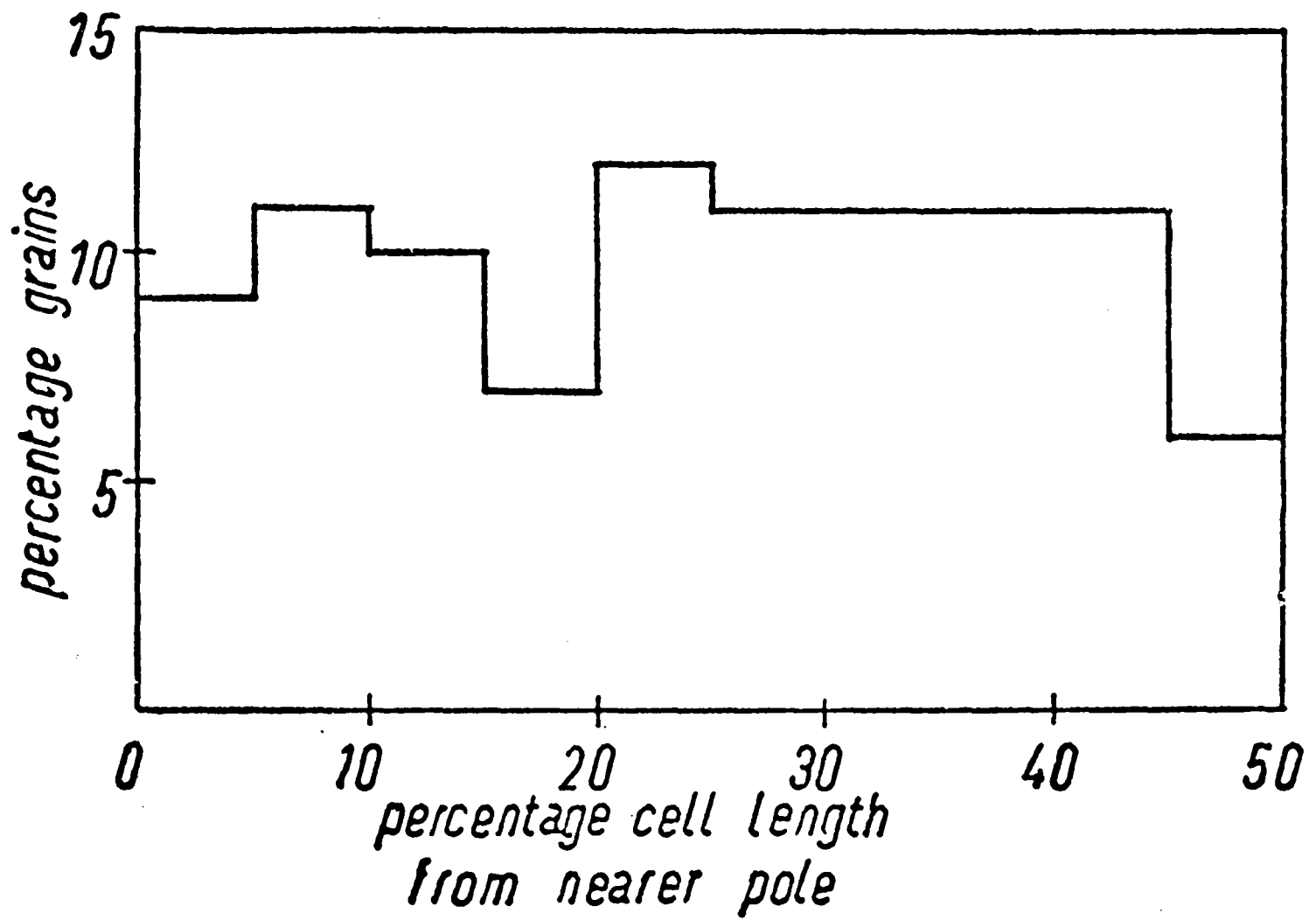
The previous experiment was repeated using B/r cells and with one added feature. After filament formation the culture

FIGURE 4



Distribution of grains (102 grains measured) on the surface of cell "ghosts" of E.coli. B/r . Cells were pulse labelled with (3H) histidine for 5 min. and subsequently grown for two mass doublings in the presence of penicillin 30 I.U./ml. Data expressed as in Fig. 1.

FIGURE 5



Distribution of grains (364 grains measured) on the surface of cell "ghosts" of E.coli. KM7. Cells were grown for two mass doublings in the presence of penicillin 60 I.U./ml. before being pulse labelled with (3H) histidine for 1 min. immediately prior to ghost preparation.

was split into two aliquots. One was pulsed immediately with ^3H histidine and used immediately for ghost preparation, the other was treated with penicillinase (Wellcome Laboratories) for 10 min prior to histidine pulsing to restore its septum forming capacity. There is a twenty minute gap (approximately), between inactivation (or removal) of penicillin and resumption of division (126 and unpublished observation). The object of this procedure was to make the labelling conditions more analogous to those of Ryter et al (30) and Schwarz et al (93) when they demonstrated incorporation of labelled diaminopimelic acid (DAP) at the site of the forming septum.

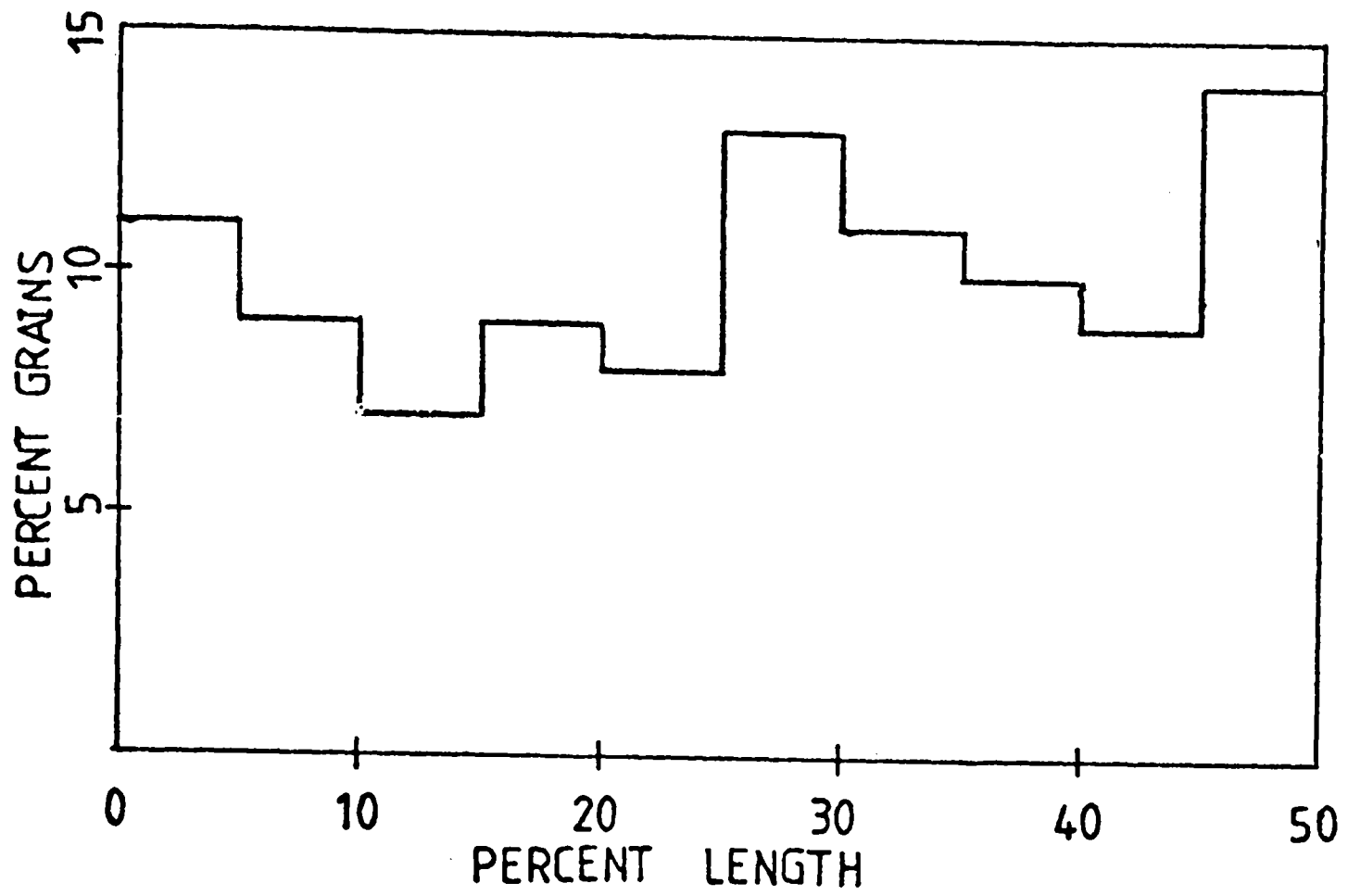
However, the grain distribution in both cases was clearly random. See Figures 6a and 6b, also 7a and 7b. The possibility that the random distributions, observed in the above experiments, were the result of an artefact was investigated by determining whether or not matrix protein/proteins dissociated and reassociated during ghost preparation.

Equal proportions (mass) of penicillin induced filaments labelled with ^3H histidine and unlabelled normal-sized cells of E.coli KM7 were mixed immediately before ghost preparation. Autoradiograms were prepared from the mixed ghost preparation and showed that the radio-active label was confined to the filaments indicating no dissociation and reassociation of matrix protein between ghosts or cells.

Discussion

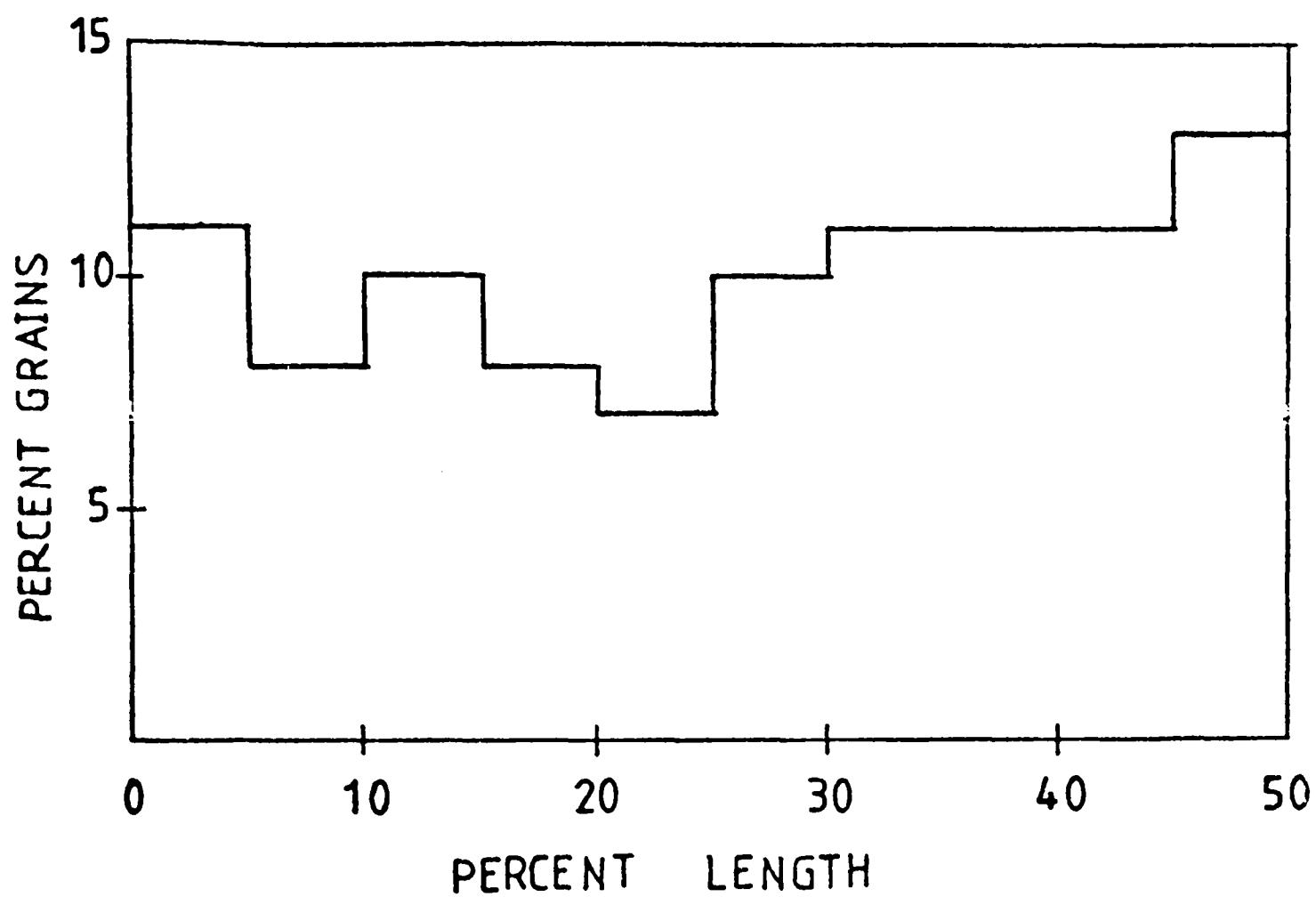
The investigation into insertion and distribution of matrix protein suggests that matrix protein is not spatially conserved in the cell envelope, and becomes distributed at random over the cell surface during cell growth. In addition,

FIGURE 6a



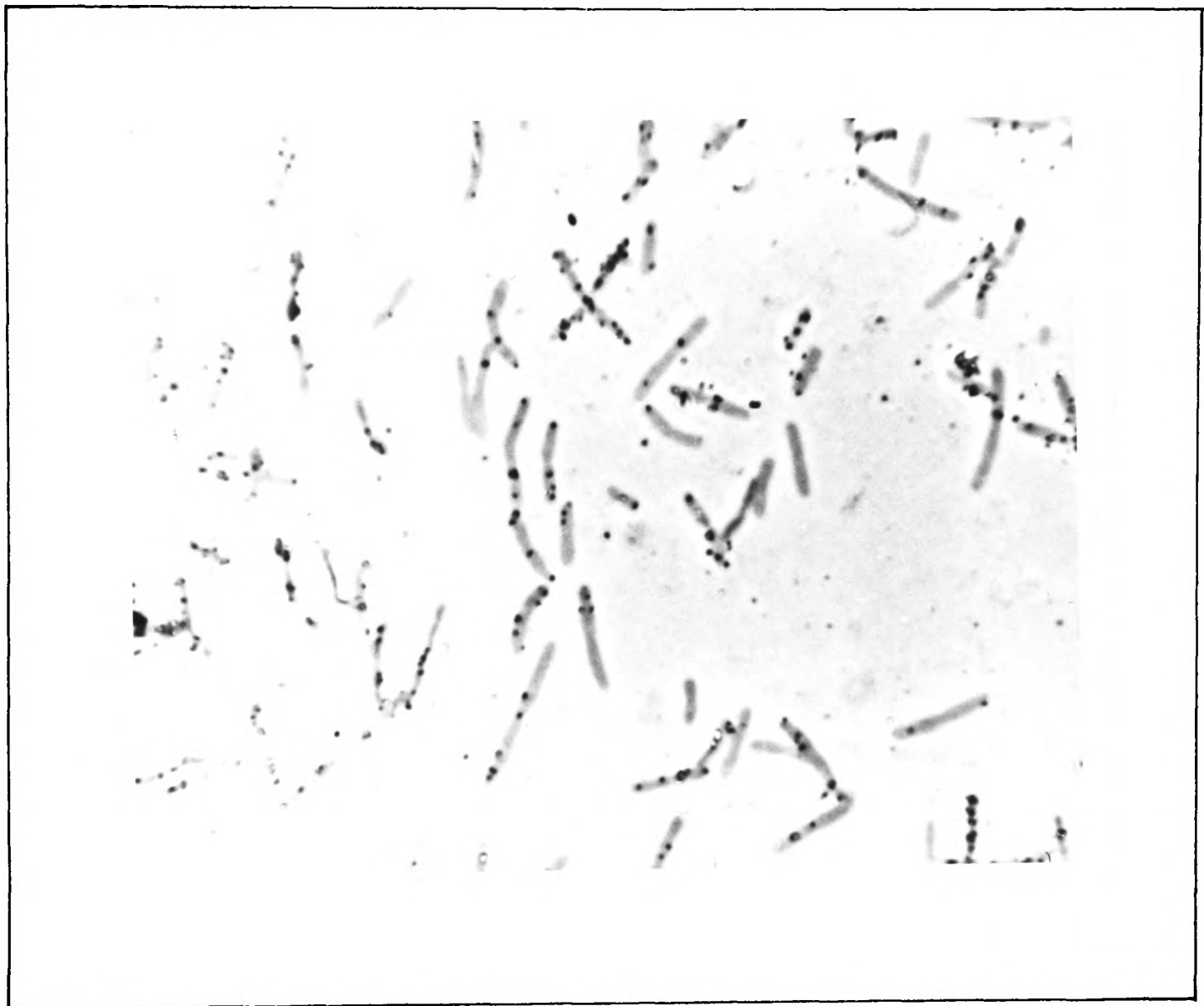
Distribution of grains (360 grains measured) on the surface of cell ghosts of *E. coli*. B/r. Cells were grown for two mass doublings in the presence of penicillin 30 I.U./ml. before being pulse labelled with (3H) histidine for 1 min. immediately prior to ghost preparation.

FIGURE 6b



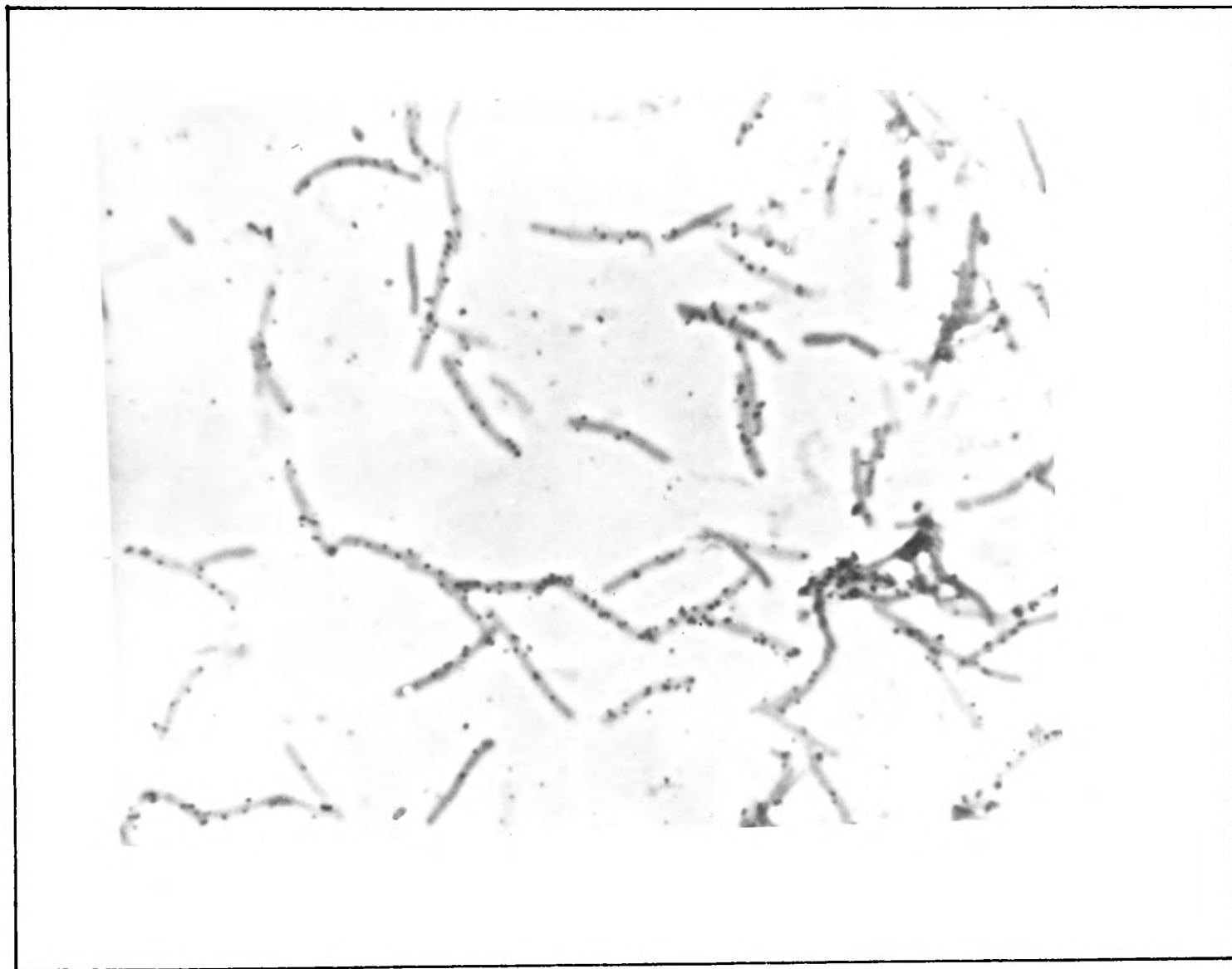
Distribution of grains (379 grains measured) on the surface of cell ghosts of *E. coli*. B/r. Cells were treated as in the legend to Fig. 6a but were given a 10 min. treatment with penicillinase prior to radio-active labelling.

FIGURE 7a.



Photomicrograph of ghosts described in Fig. 6a. X 1500.

FIGURE 7b.



Photomicrograph of ghosts described in Fig 6b. X 1500.

the experiments with pre-formed filaments suggest that it is not even localised for short periods of time at the instant of incorporation. Although it has been demonstrated that matrix protein does not dissociate and reassociate, there remains a possibility that randomising of the label is the result of a procedural artefact which causes relocation of matrix protein within individual cells.

It has been shown that mutant cells of E.coli which lack matrix protein can exist and grow quite normally without it (127,128). This observation, together with the report that the proportion of matrix protein and other outer membrane proteins can vary with cultural conditions (50), and the above results, lead to the conclusion that matrix protein is not part of any conserved structure in the cell envelope.

Smit and Nikaido (129) have recently reached similar conclusions in their study on the porin proteins of Salmonella typhimurium. These authors have shown that these porin proteins are inserted all over the cell surface, possibly at the areas of adhesion between the inner and outer membranes described by Bayer (130). (The porin proteins of Salmonella are analogous to the matrix proteins of E.coli and the term porin is often applied to the matrix proteins of E.coli (46).)

B. An investigation into the insertion of bound murein lipoprotein

Although it is known that murein lipoprotein is subject to rapid turnover (131), and is therefore an unsuitable subject for long term distribution studies, it was thought that a study on the mode of insertion of the bound form of such an abundant protein might be of value.

- (1) Cells of E.coli B/r were induced to filament by growing them in the presence of penicillin for two mass doublings. The cells were pulse labelled with ^3H leucine for one minute immediately prior to sacculi preparation. Autoradiograms were prepared.
- (2) Filaments prepared as in (1) were treated with penicillinase for ten minutes prior to pulse labelling and preparation of sacculi as in (1).

These two experiments were repeated using E.coli KM7 cells.

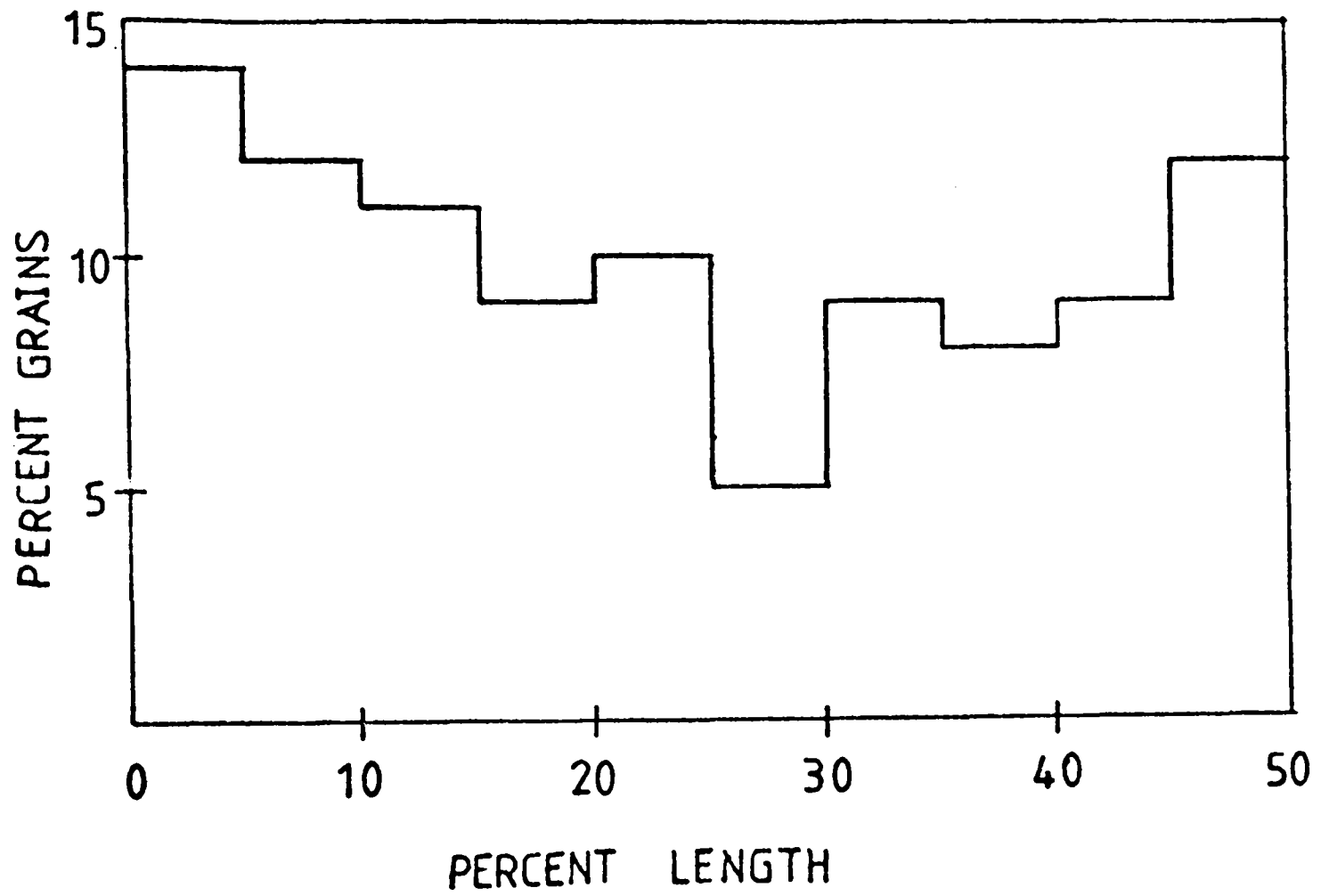
In all cases the grain distribution pattern was random. Figures 8a and 8b show the grain distributions for the B/r cells.

The protein content of the sacculi was analysed by disrupting the mucopeptide with lysozyme at 200 $\mu\text{g/ml}$, then analysing the suspensions on polyacrylamide gels. The only bands observed were one well defined band at around 14,000 molecular weight (the lysozyme) and a hazy ill defined band near the bottom of the gel which was consistent with the appearance of lipoprotein on 16% polyacrylamide gels (J. Lutkenhaus, personal communication). This information combined with the fact that sacculi from cells labelled with radio-active histidine were unlabelled - see section A - and sacculi from cells labelled with radio-active leucine had an abundance of labelled protein attached, was taken as a strong indication that the protein under study in this section was indeed murein lipoprotein.

Discussion

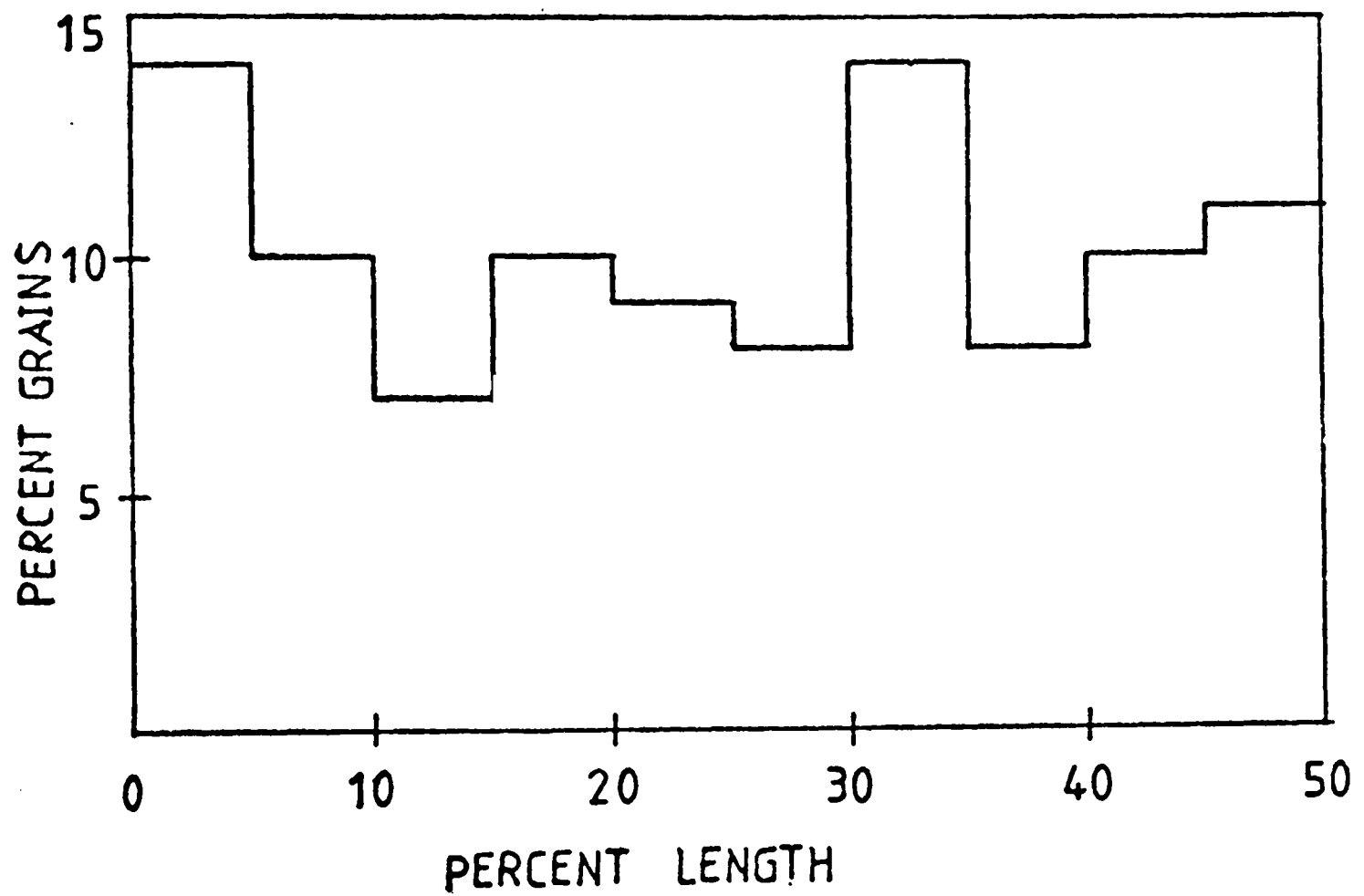
It was thought possible that a study on the insertion of murein lipoprotein might have yielded confirmatory evidence

FIGURE 8a



Distribution of grains (338 grains measured) on the surface of cell sacculi of *E. coli*. B/r. Cells were grown for two mass doublings in the presence of penicillin 30 I.U./ml. before being pulse labelled with (3H) leucine for 1 min. immediately prior to sacculi preparation.

FIGURE 8b



Distribution of grains (320 grains measured) on the surface of cell sacculi of *E. coli*. B/r. Cells were treated as in the legend to Fig. 8a but were given a 10 min. treatment with penicillinase prior to radio-active labelling.

to the work of Ryter et al (30) and Schwarz et al (93) who proposed that new murein was incorporated at the centre of the cell. This has not been the case. The evidence presented in this section suggests that bound murein lipoprotein is inserted at random over the entire surface of the cells. This evidence however, cannot be taken as a direct contradiction of the Ryter-Schwarz proposition. The free form of lipoprotein is presumed to be the precursor of the bound form (131) but there is no evidence to suggest that this free lipoprotein is bound to new murein at the time of its incorporation. There may in fact be a delay before covalent bonding of lipoprotein takes place, which would mean that the insertion of bound lipoprotein would not necessarily reflect the mode of insertion of new murein. On the other hand it has been suggested that invagination of the outer membrane during septum formation involves covalent linking of free lipoprotein to the murein of the nascent septum (132). This proposition was based on the observation that mutants with a defect in the formation of murein bound lipoprotein showed defective invagination of the outer membrane during cell division. If this were true, one might reasonably have expected to find a higher concentration of label at prospective division sites on the filaments which had been treated with penicillinase prior to labelling. This was not the case, but it is possible that there is a delay between insertion as free lipoprotein and conversion to the bound form.

C. Studies on mutants lacking major outer membrane proteins

It was believed that the "ghost" technique described by Henning et al (28) might be used on mutant strains of E.coli, which lacked certain major outer membrane proteins, to

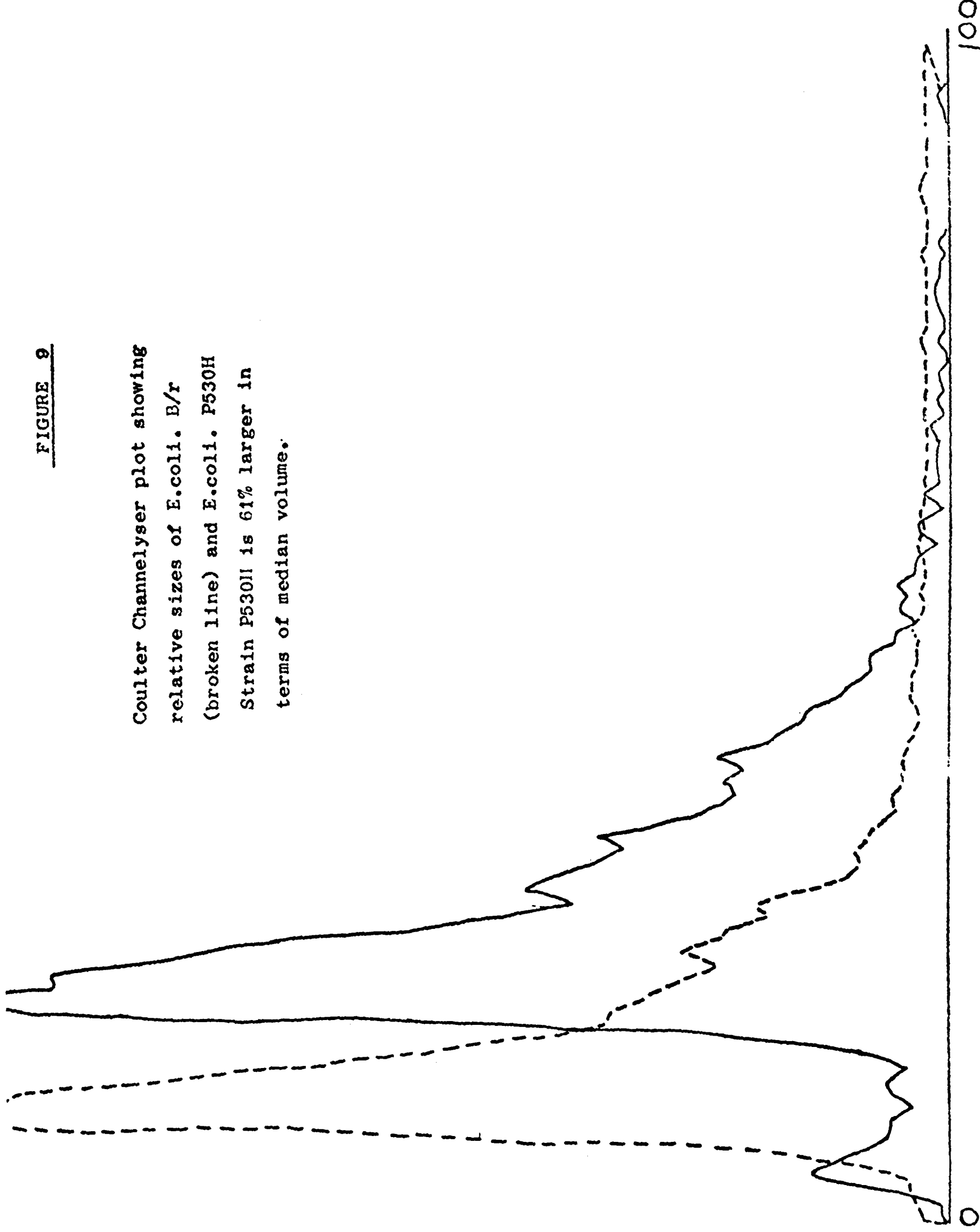
Provide information about the localisation of other major outer membrane proteins.

Early attempts to produce well formed rod shaped ghosts from mutants lacking matrix proteins Ia and Ib were generally unsuccessful; the ghosts proving to be very "fragile" with a tendency to collapse completely. In an attempt to increase stability of the ghosts, I decided to modify the Henning procedure in such a way as to retain the sacculi. This was simply achieved by omitting the lysozyme treatment step in the protocol. This modification proved to be very successful and it was now possible to produce well shaped cell ghosts from strains which lacked matrix protein. Four such mutants were tested for ghost production using the modified protocol and it was decided that E.coli P530H (derived from the well characterised ompB strain (133) P530 (114)), which was completely devoid of proteins Ia and Ib, would be used in subsequent experiments. This decision was made for two reasons, (1) P530H produced a high yield of well formed, rod shaped ghosts, (2) P530H is a larger than average strain of E.coli, which would permit better resolution of grain position during autoradiography (see Figure 9).

A second problem arose when estimating the best concentration of penicillin to use for filament induction. It appeared that P530H was unduly sensitive to penicillin at the concentrations normally used for this purpose (15-60 IU/ml) (104,116). Reducing the concentration however, did not improve matters and it seemed that there was no "middle ground" between a concentration which was ineffectual and one which lysed the cells. This problem was resolved by using another β -lactam antibiotic - Cephalexin (123). Cephalexin proved to

FIGURE 9

Coulter Channelyser plot showing
relative sizes of E.coli. B/r
(broken line) and E.coli. P530H
Strain P530H is 61% larger in
terms of median volume.



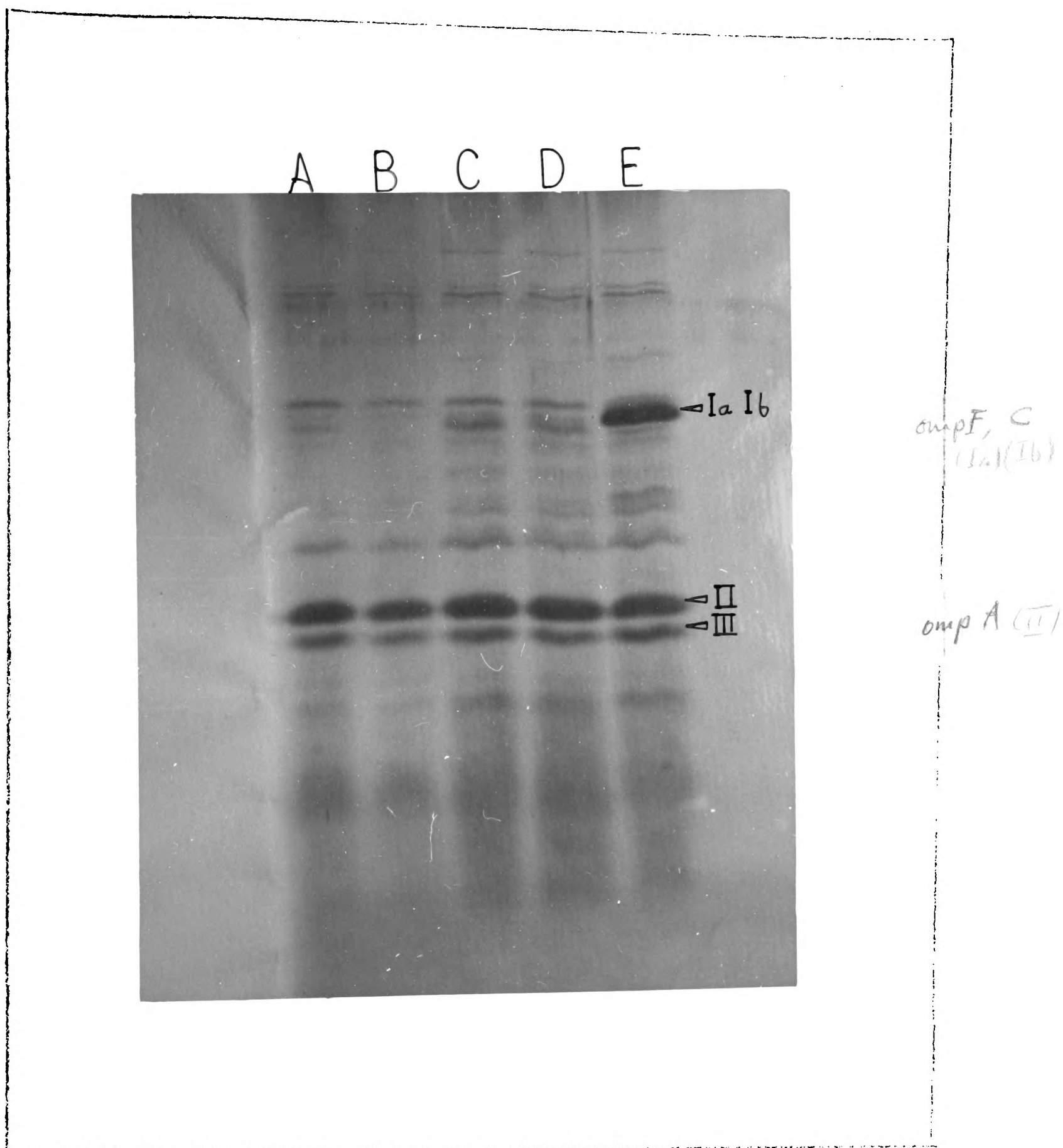
be extremely efficient at producing filamentation without lysis. Its one drawback appeared to be that it slowed cell growth rate immediately on addition.

Preliminary characterisation of the protein content of cell ghosts produced by the modified Henning technique was carried out by analysing many ghost preparations on 16% polyacrylamide gels. It was found that the protein pattern was reproducible and unchanged when cells were treated with cephalixin. E.coli P400 was used for this preliminary study - this being the parent strain of P530H.

The gel pattern showed one dense band at molecular weight $\sim 36,000$ (Ia and Ib are not separated on the gel system used), a second dense band at m.w. $\sim 19,000$, a third less dense band at m.w. $\sim 18,000$ and several minor bands. Bound lipoprotein would not enter the gel as the sacculi had not been disrupted by lysozyme but free lipoprotein may have been responsible for the low molecular weight smudge at the bottom of the gel. As it was planned to use radio-active histidine in the experiments the presence or absence of lipoprotein (which contains no histidine) was of little importance with regard to uptake of label. Ghosts prepared from P530H gave identical gel patterns to P400 with the exception that the dense band at m.w. 36,000 was missing. Figure 10 shows PAGE analysis of two separate ghost preparations from P530H cells treated with cephalixin, two separate ghost preparations from untreated P530H cells, and one preparation from untreated P400 cells.

The presence of a dense gel band at m.w. $\sim 19,000$ was puzzling and in the early stages of this work it was thought that this band was in fact protein III (m.w. $\sim 18,000$). This

FIGURE 10

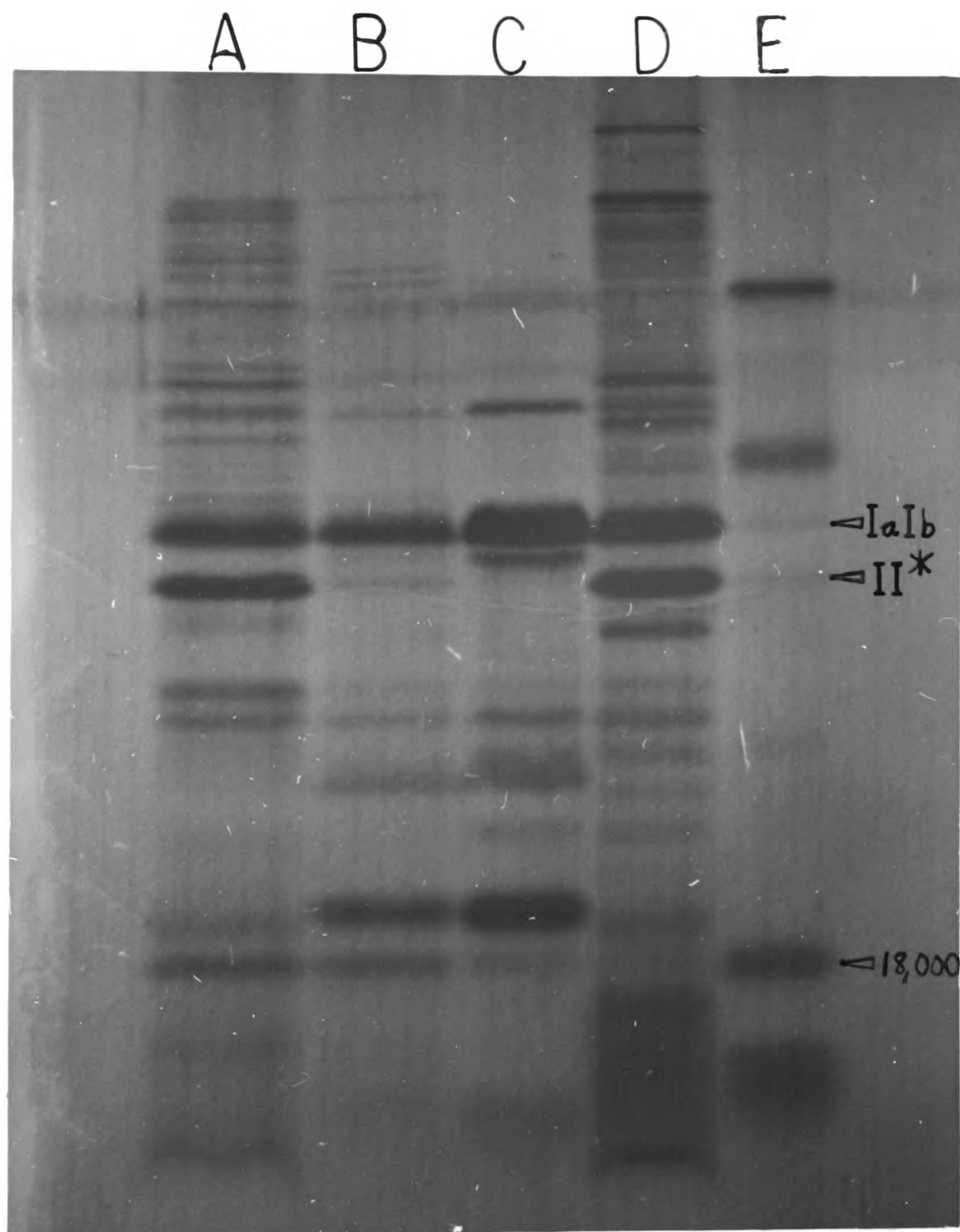


Stained polyacrylamide gel after electrophoresis of proteins from cell ghosts of :- A and B, P530H cells treated and untreated with cephalixin respectively. C and D, repeat of A and B on a different day showing the constant nature of the protein pattern.
E. P400 cells untreated.

however meant that protein II - the tryptic fragment of protein II* was missing from all the ghost preparations and the absence of such a major protein component was extremely worrying. The band at m.w. $\sim 19,000$ however, was so dense that a suspicion arose that this band was in fact protein II running at a different molecular weight from that previously reported - 28,000 in the original paper (28) and 24,000 in a later investigation (134). This possibility was investigated by analysing cell envelope preparations, before and after trypsin treatment, on polyacrylamide gels. In addition Henning's ghost preparations were analysed in similar fashion before and after the stage involving trypsin treatment. In all cases the dense band at m.w. 33,000 (protein II*) disappeared after treatment with trypsin and a new dense band appeared at m.w. 19,000. (See Figure 11). Further evidence that the dense band at m.w. 19,000 was the tryptic fragment of protein II* was obtained from strain P530HK3, a variant of P530H which lacks protein II* as well as Ia and Ib. Cell envelope preparations from this strain had no dense band at m.w. 33,000 and Henning's ghost preparations had no dense band at m.w. 19,000. Figure 12 shows PAGE analysis of cell envelopes and Henning's ghost preparations from the three strains in use in this section, i.e. P400 the parent, P530H lacking Ia and Ib, and P530HK3 lacking Ia, Ib and II*.

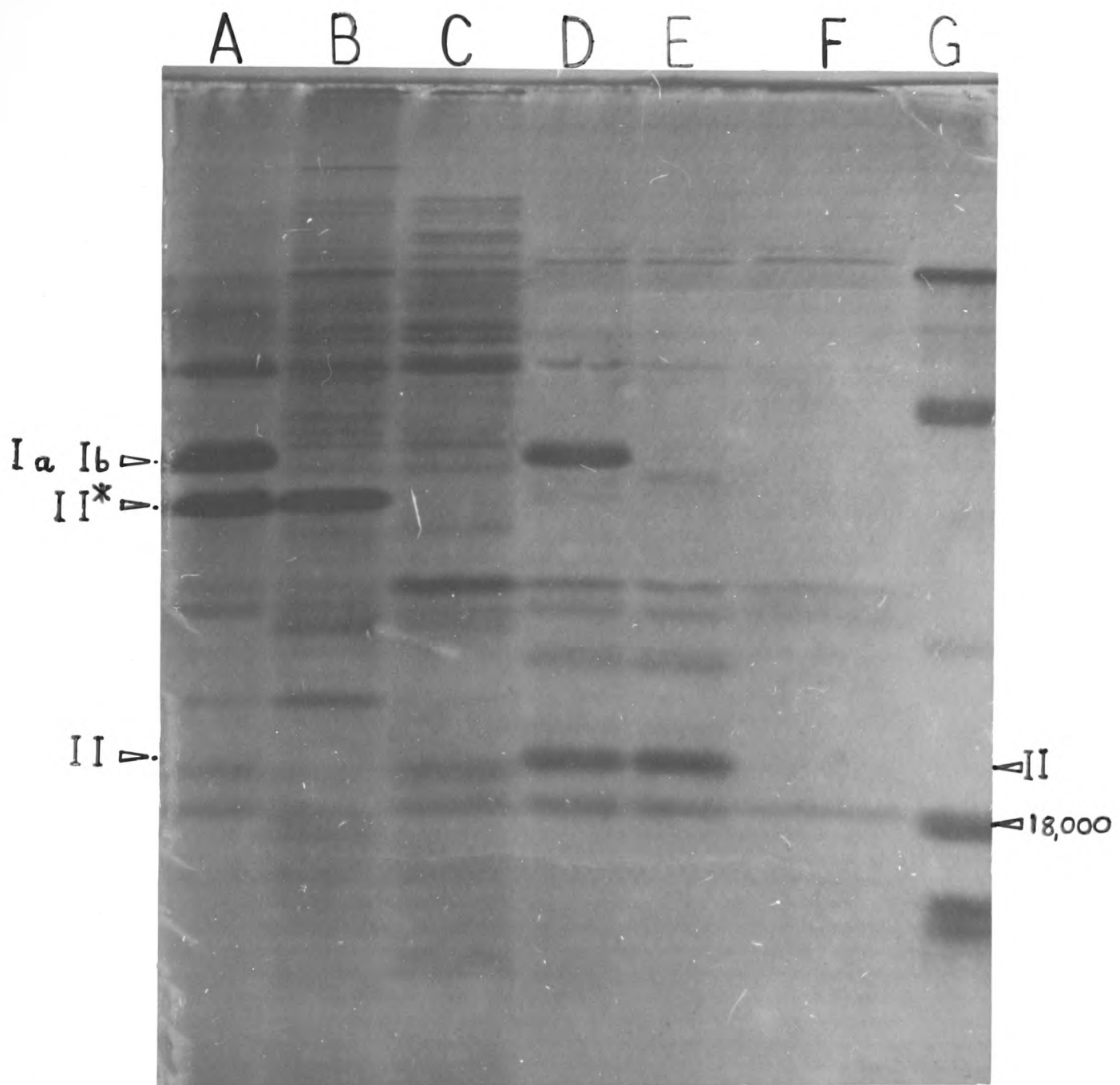
To check for possible contamination of the trypsin, a cell envelope preparation of P400 was treated with three different brands of trypsin. In all cases the tryptic fragment of protein II* appeared at m.w. $\sim 19,000$. No explanation can be offered for the difference in molecular weight of the fragment in this work, compared to the reported value, other

FIGURE 11



Stained polyacrylamide gel showing :- A, cell envelopes of P400
B, cell envelopes of P400 after trypsin treatment C, a ghost
preparation of P400 after trypsin treatment D, the same preparation
before trypsin treatment E, molecular weight standards, in
descending order from the top 68,000, 42,000, 18,000, 14,000, 12,700.
The position of proteins Ia, Ib, II* and the 18,000 molecular
weight standard : is indicated.

FIGURE 12



Stained polyacrylamide gel showing:- Cell envelope proteins of A, P400 B, P530H C, P530HK3. Henning ghost preparations from D, P400 E, P530H F, P530HK3. G, shows molecular weight standards as in Fig. 11. The positions of proteins Ia, Ib, II*, and II (tryptic fragment of II*) are indicated along with the 18,000 molecular weight standard.

than the fact that reported molecular weights for proteins on SDS polyacrylamide gels tend to vary a great deal from one laboratory to another. Protein II* has been reported as having a molecular weight varying from 27,000 to 36,000 before heat treatment and 33,000 to 48,000 after heat treatment (52 and references therein).

The larger than normal size of strain P400 and derivatives, together with improved autoradiographic techniques, gained through experience with the earlier experiments in this thesis, made it feasible to attempt a series of experiments on normal sized cells, i.e. without the use of inhibitors of division.

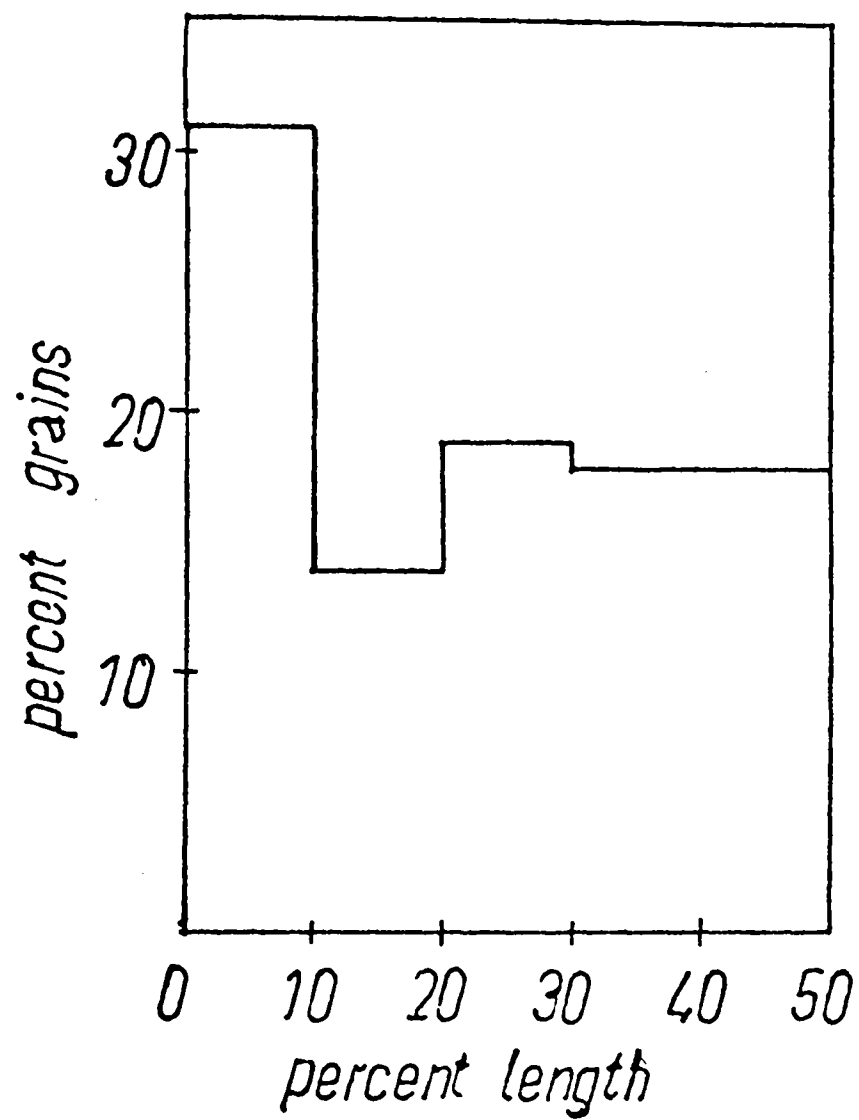
Strain P400 was grown to steady state in a minimal medium supplemented with glucose and casamino acids. The cells were washed and resuspended in fresh minimal medium containing glucose, required amino acids, and radio-active histidine. After a ten minute pulse Henning ghosts were prepared and autoradiograms made. The resultant grain distribution is predominantly random - see Figure 13.

NOTE In previous histograms of grain distributions the cells have been apportioned in 5% length fractions. In Figure 13 however, and indeed all experiments involving normal sized cells, 10% length fractions are used. This is a more realistic figure for cells of such length and keeps measurements within the limits of resolution of the light microscope.

The predominantly random distribution shown in Figure 13 is of course expected as matrix protein is present.

Figure 14 shows the grain distribution obtained when strain P530H was treated exactly as P400 in the previous

FIGURE 13



Distribution of grains (188 grains measured) as a function of cell length in cell ghosts of E.coli. P400. Cells were pulse labelled with (3H) histidine for 10 min. immediately before ghost preparation.

experiment. This time the grain distribution is non-random and exhibits a marked degree of polar labelling. See also Figure 14A.

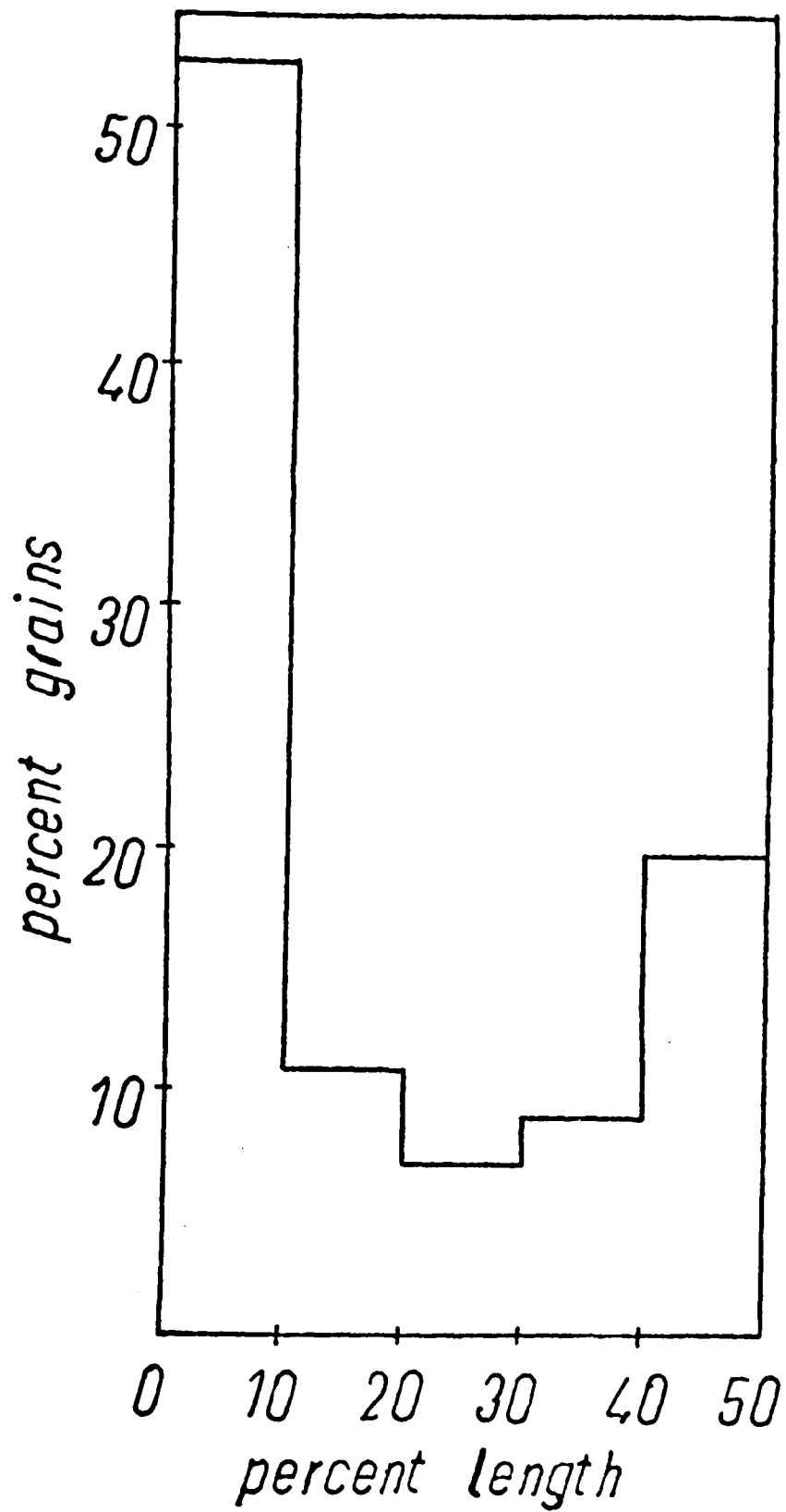
This result was both surprising and exciting and it was tempting to assume that the strong polar label was due to the tryptic fragment of protein II* which is certainly the most abundant protein in ghosts prepared from P530H - see Figure 10. This protein however is obviously not the only one present in the ghosts and indeed the physical amounts of the proteins in this instance are not as relevant as the histidine content of the various proteins. It was decided to investigate the cause of the polar labelling pattern by 1) Performing the above autoradiographic experiment using strain P530HK3 which lacks matrix protein and protein II* and would therefore provide ghosts identical to P530H except that the II* fragment would be missing. 2) Estimate the relative amounts of radio-active label in the II* fragment compared to the other proteins in P530H ghosts.

Figure 15 shows the grain distribution from the experiment with P530HK3. The distribution is random in the absence of the II* fragment.

Radio-active protein analysis performed by gel slicing, solubilising, and counting as described in Materials and Methods demonstrated that approximately 40% of the total label present in cell ghosts of P530H was contained in the protein II* fragment.

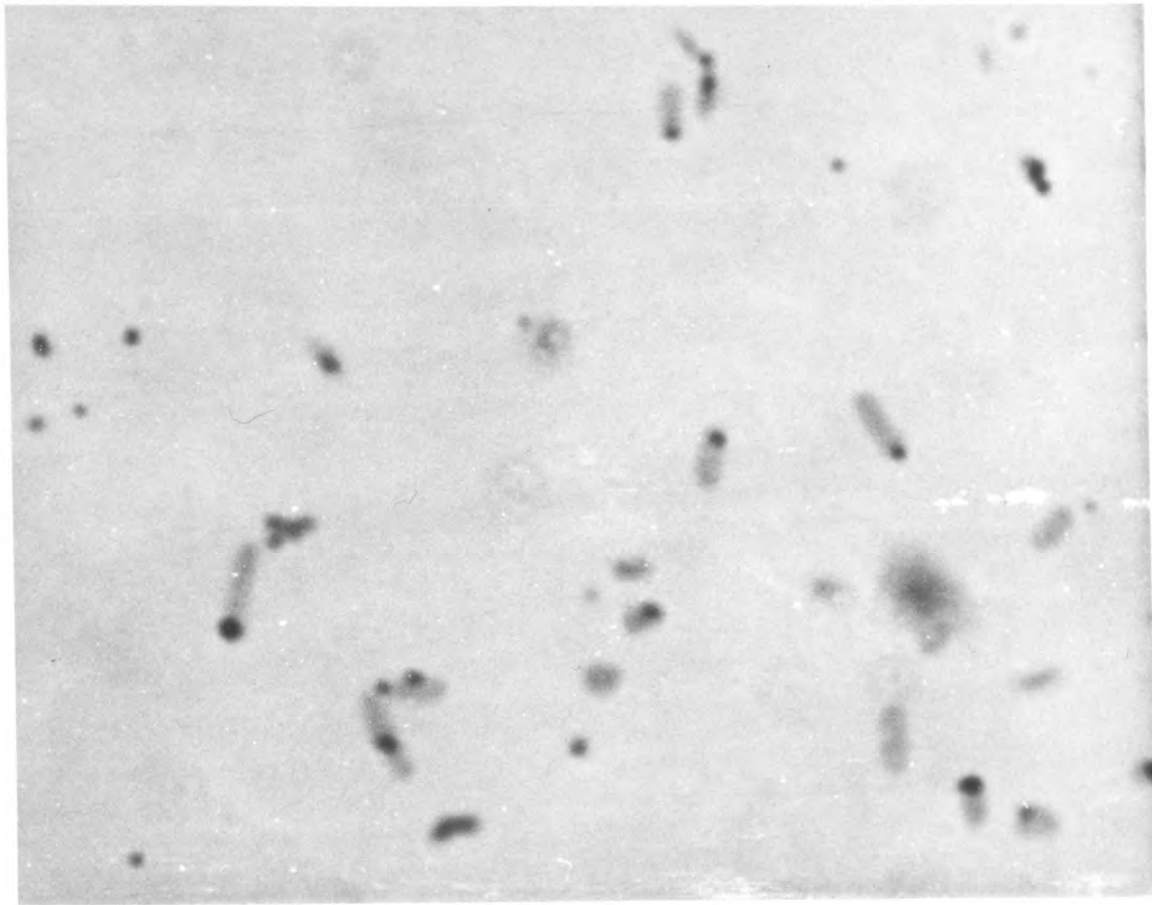
It seemed clear from the above results that the II* fragment did indeed contain radio-active label and was in fact responsible, at least in part, for the polar labelling bias. Although, as has been stressed, this protein is a

FIGURE 14



Distribution of grains (278 grains measured) in cell ghosts of P530H. Cells were pulse labelled with (3H) histidine for 10 min. immediately before ghost preparation.

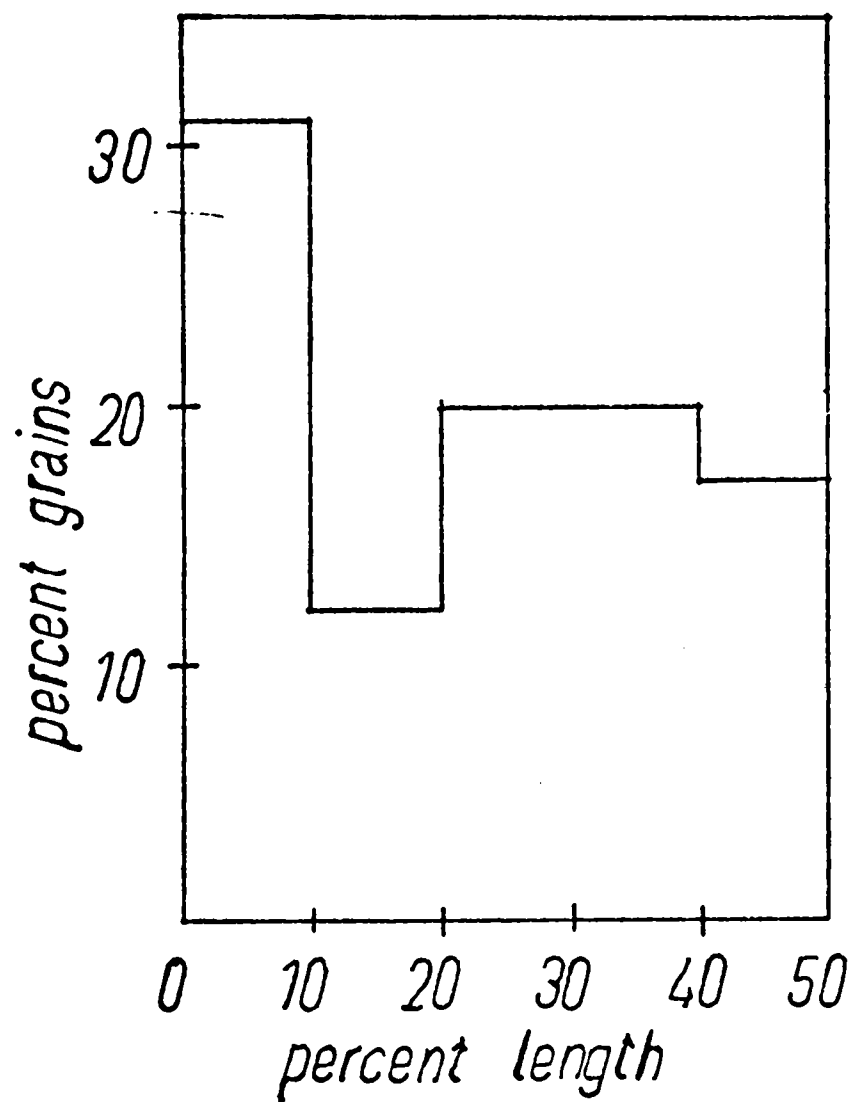
FIGURE 14a



Photomicrograph of ghosts described in Fig. 14.

x 2,400.

FIGURE 15



Distribution of grains (309 grains measured) in cell ghosts of E.coli. P530HK3. Cells were pulse labelled with (3H) histidine for 10 min. immediately before ghost preparation.

tryptic fragment of the major outer membrane protein II*, it was felt that the behaviour of the fragment in these studies might well reflect the behaviour of the II* protein itself. The fragment has been studied in some detail by Schweizer et al (134). These workers found that the fragment exhibited the same phage receptor activity as the complete protein in the presence of lipopolysaccharide. As lipopolysaccharide is only found at the outer surface of the outer membrane (135) it seems that the tryptic fragment represents the outer part of a protein which has been shown to be trans-membrane (56).

The investigation was continued by pulse labelling cells of P530H as before, chasing with non radio-active histidine, and allowing growth and division to continue for one mass doubling. The grain distribution is shown in Figure 16. Again the distribution is clearly non-random. There is a marked concentration of labelled protein at the poles of the ghosts.

NOTE This experiments performed with P530HK3 (i.e. lacking II*) gave a random grain distribution.

Figures 17a and 17b show the result of pulse labelling P530H cells with radio-active histidine, chasing with non radio-active histidine and allowing growth to continue in the presence of cephalixin to inhibit division. This time the labelled protein appears to be concentrated at the poles and regions which would be septal sites if the filaments were allowed to divide, i.e. 25% and 50% of the cell length.

In view of the above results the possibility that protein II* could migrate into polar and septal sites was considered. It was decided to investigate the labelling pattern when

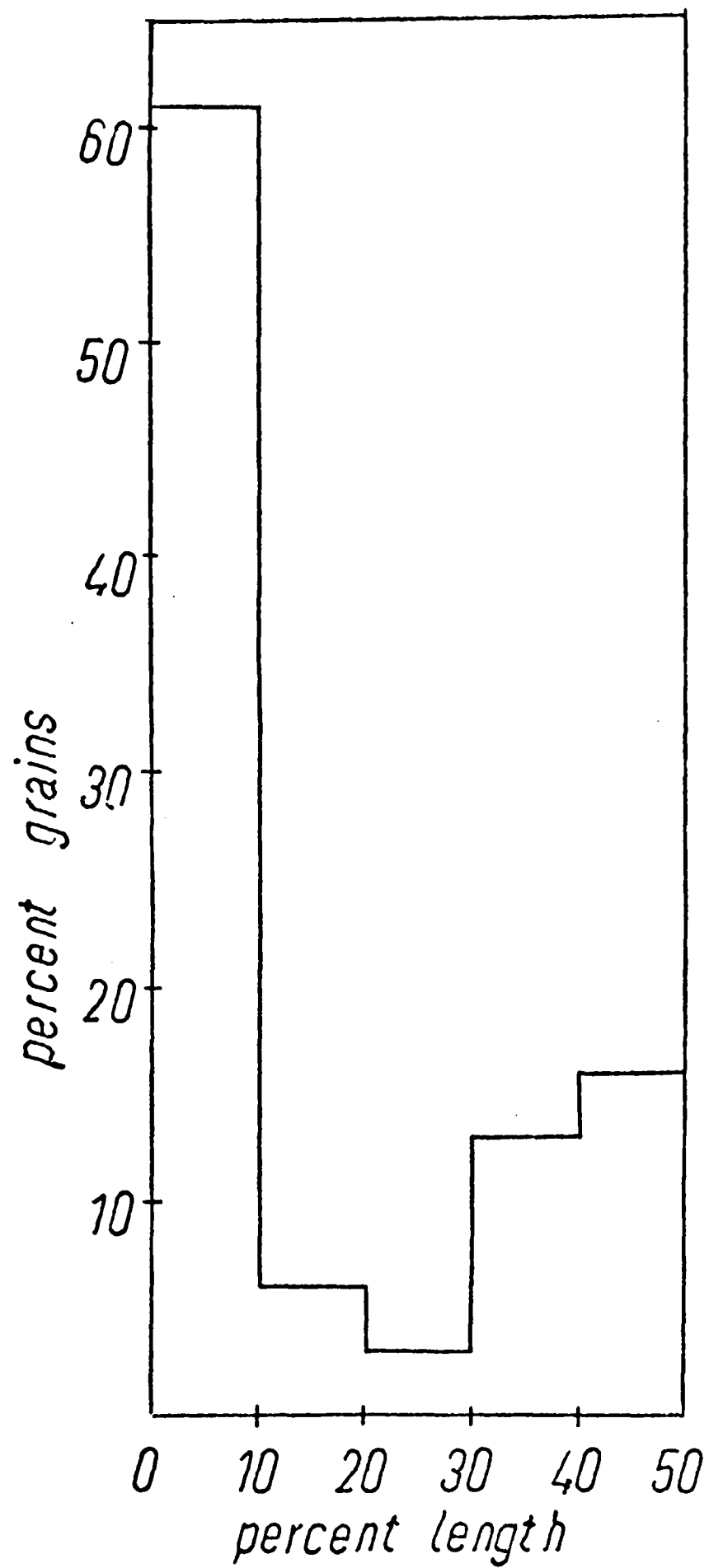
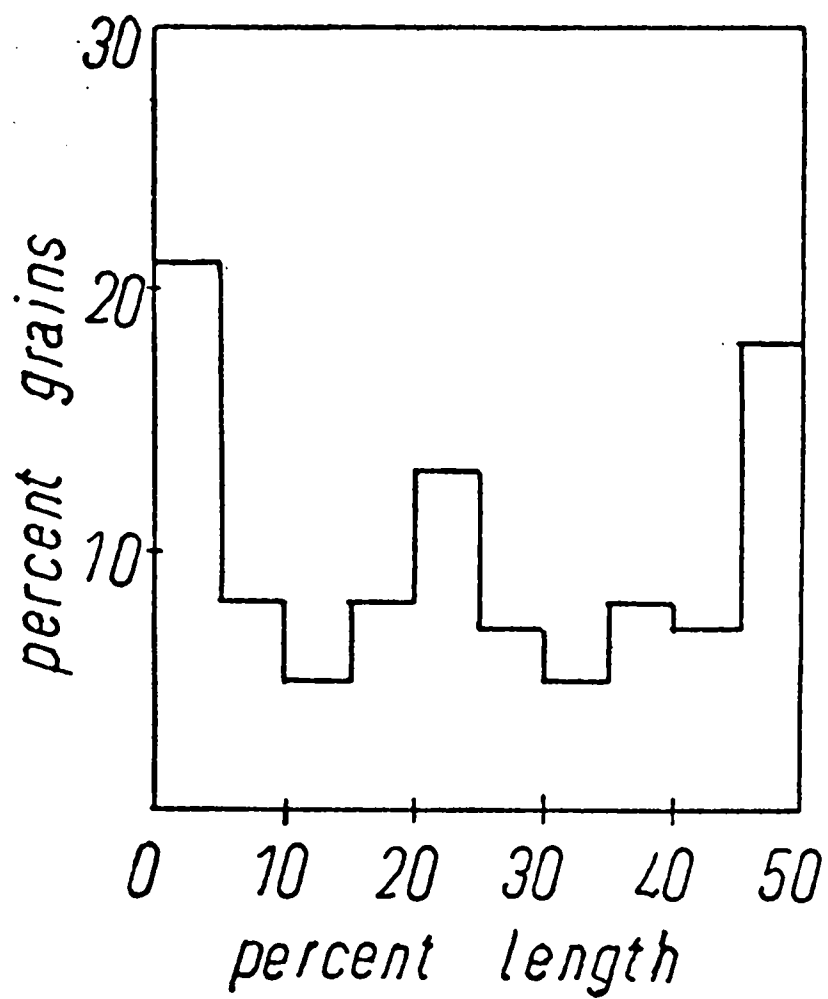


FIGURE 16

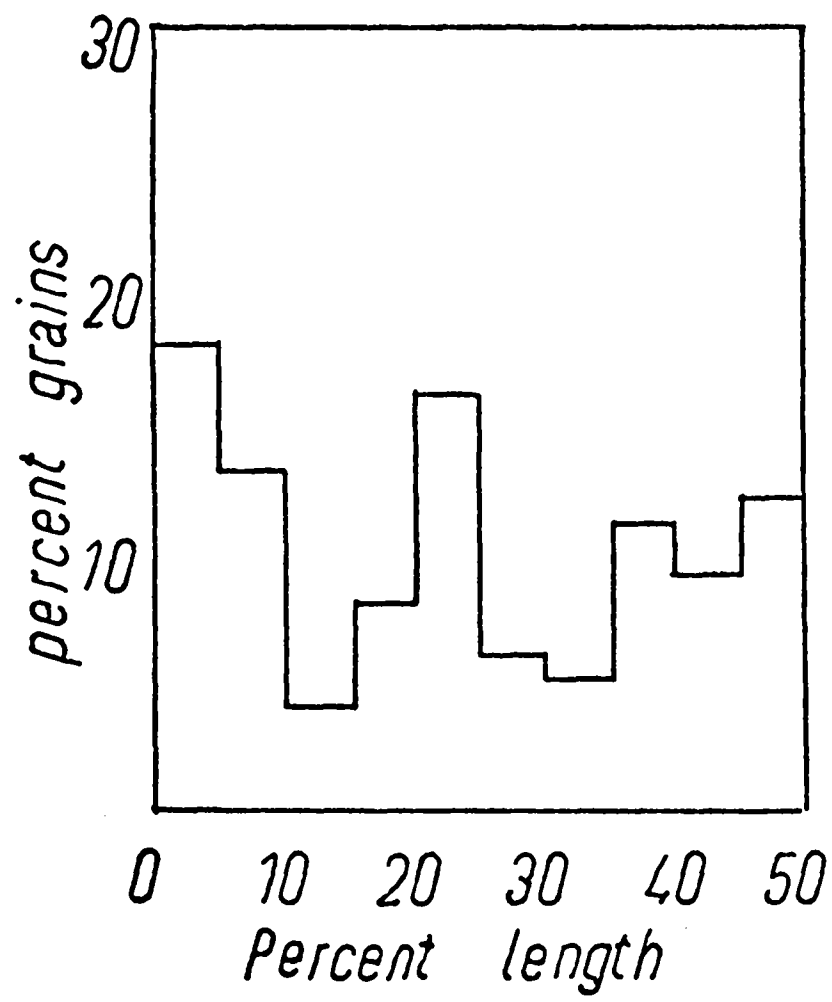
Distribution of grains (330 grains measured) in cell ghosts of E.coli. P530H. Cells were pulse labelled for 10 min. with (3H) histidine, chased with non-radioactive histidine and allowed to grow and divide for one mass doubling.

FIGURE 17a



Distribution of grains (336 grains measured) in cell ghosts of P530H. Cells were pulse labelled with (3H) histidine for 10 min., chased with non-radioactive histidine, and allowed to grow for one mass doubling in the presence of cephalixin to inhibit division.

FIGURE 17b



Cells treated as in Fig. 17a but grown for two mass
doublings in the presence of cephalixin.
(342 grains measured)

filaments of P530H induced by cephalixin, were pulsed with ^3H histidine immediately prior to ghost preparation.

Figure 18 shows the grain distribution resulting from this experiment. Somewhat surprisingly the distribution is completely random when it had been expected that a polar and septal concentration of labelled protein would have been obtained.

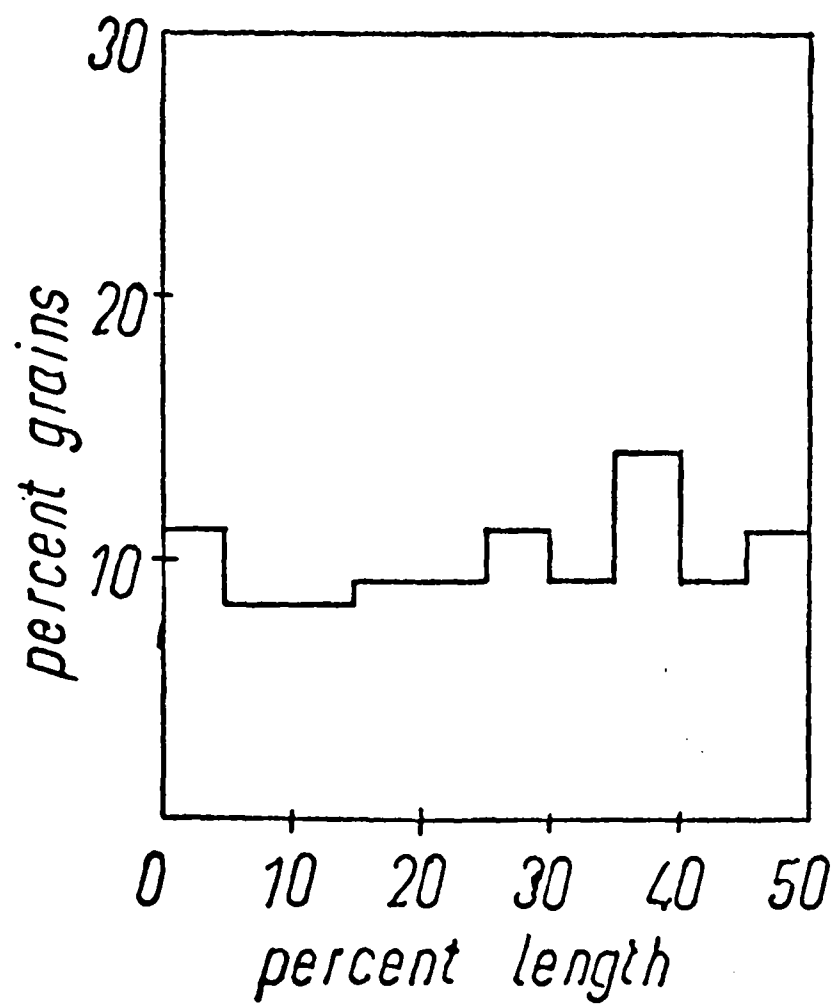
The possibility that the presence of cephalixin might have been responsible for the random distribution arose though making the proposition that if cephalixin inhibited the septation process then it might conceivably inhibit location of any protein normally (hypothetically) concentrated at septal sites.

This proposition was tested in the following manner. Cells of P530H were induced to filament as in the previous experiment. After pulse labelling in the presence of cephalixin the culture was split into two. One half was used for ghost preparation immediately, the other had the cephalixin removed by filtering and washing. These cells were allowed to continue growing for ten minutes before ghosts were prepared.

NOTE Cell division re-starts between thirty and forty minutes after removal of cephalixin in this strain under these experimental conditions.

This preliminary experiment gave an encouraging result in that the cells which were pulse labelled in the presence of cephalixin and stopped immediately showed a random distribution of labelled protein as before; whereas the cells that were allowed to grow a little in the absence of cephalixin after the pulse showed a return of the polar/

FIGURE 18



Distribution of grains (488 grains measured) in cell ghosts of *E. coli*. P530H. Cells were treated with cephalixin for 1.5 mass doublings, then pulse labelled with (3H) histidine in the presence of cephalixin for 10 min. immediately before ghost preparation.

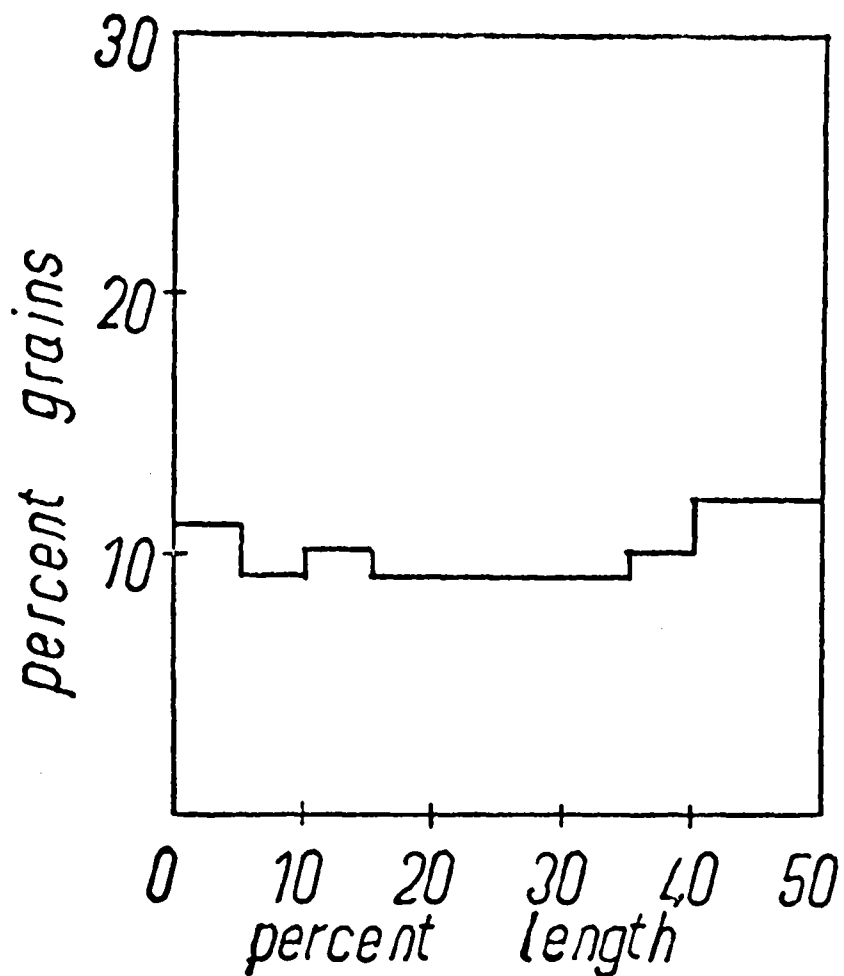
septal pattern. It was decided to repeat this experiment and extend it. The results can be seen in Figure 19. The grain distributions shown in a), b) and c) are those observed at 0 min, 15 min and 30 min respectively, after removal of cephalixin. It appears that the labelled protein which is randomly distributed at time 0 min can mobilise and relocate at polar and prospective septal sites after removal of cephalixin.

It seemed possible at this stage that inhibition of septation in general might be responsible for the lack of polar/septal concentration of protein II* (fragment). It was decided to test this hypothesis by inducing filamentation in P530H by a means other than cephalixin. Nalidixic acid, (which inhibits DNA synthesis, thereby inducing filamentation) was chosen. Cells of P530H were grown in the presence of nalidixic acid for one mass doubling before pulse labelling with ^3H histidine, still in the presence of the antibiotic. Unlike the profile obtained when labelling in the presence of cephalixin the grain distribution shown in Figure 20 shows a strong polar concentration of labelled protein. This result suggested that cephalixin might be specifically responsible for the inhibition of polar/septal concentration of the protein II* fragment.

β -lactam antibiotics inhibit several enzymes involved in the late stages of peptidoglycan metabolism (136). These enzymes can be detected in E.coli by utilising their ability to bind ^{14}C penicillin (137,138) and Spratt (139,140) has provided strong evidence that penicillin binding protein 3 (PBP3) is the target at which β -lactams interact to inhibit division. In a study on mutants temperature sensitive for

FIGURE 19

a



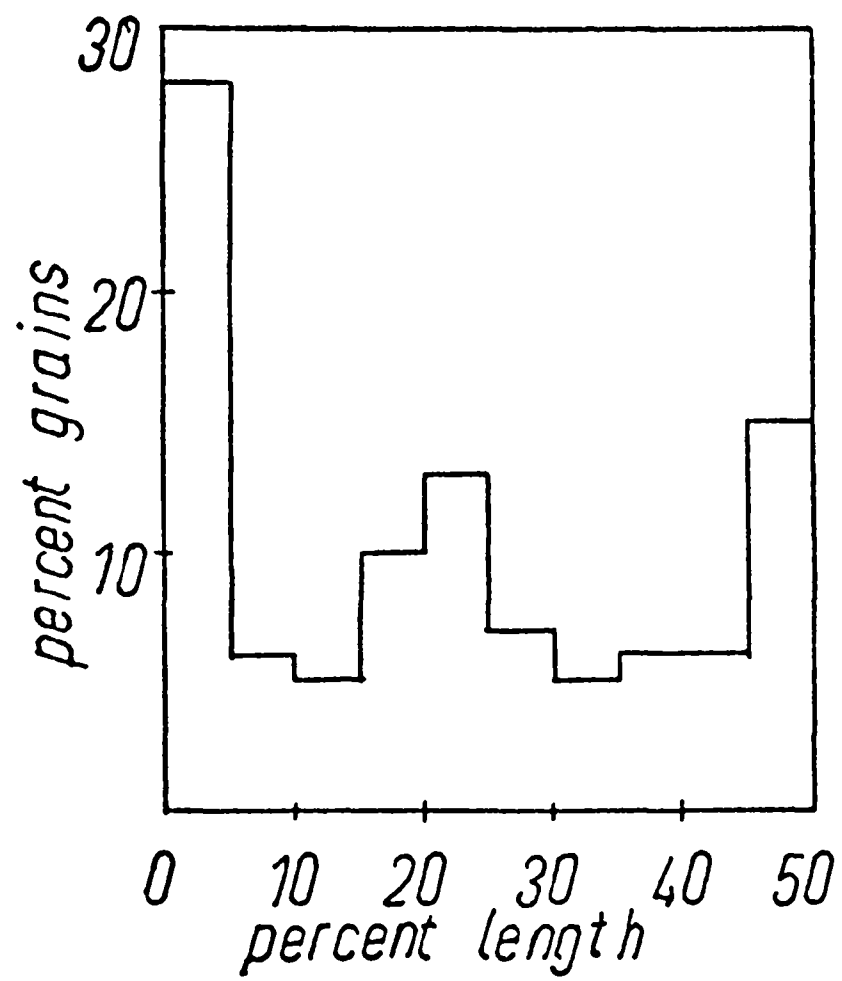
Distribution of grains obtained when cells of E.coli. P530H were treated with cephalixin for one mass doubling, pulse labelled with (3H) histidine in the presence of cephalixin for 10 min., washed free of cephalixin, and allowed to continue growing.

Grain distribution on ghosts :-

- a) 0 min. after removal of cephalixin (360 grains on 112 cells)
- b) 15 min. after removal of cephalixin (524 grains on 182 cells)
- c) 30 min. after removal of cephalixin (422 grains on 170 cells)

FIGURE 19

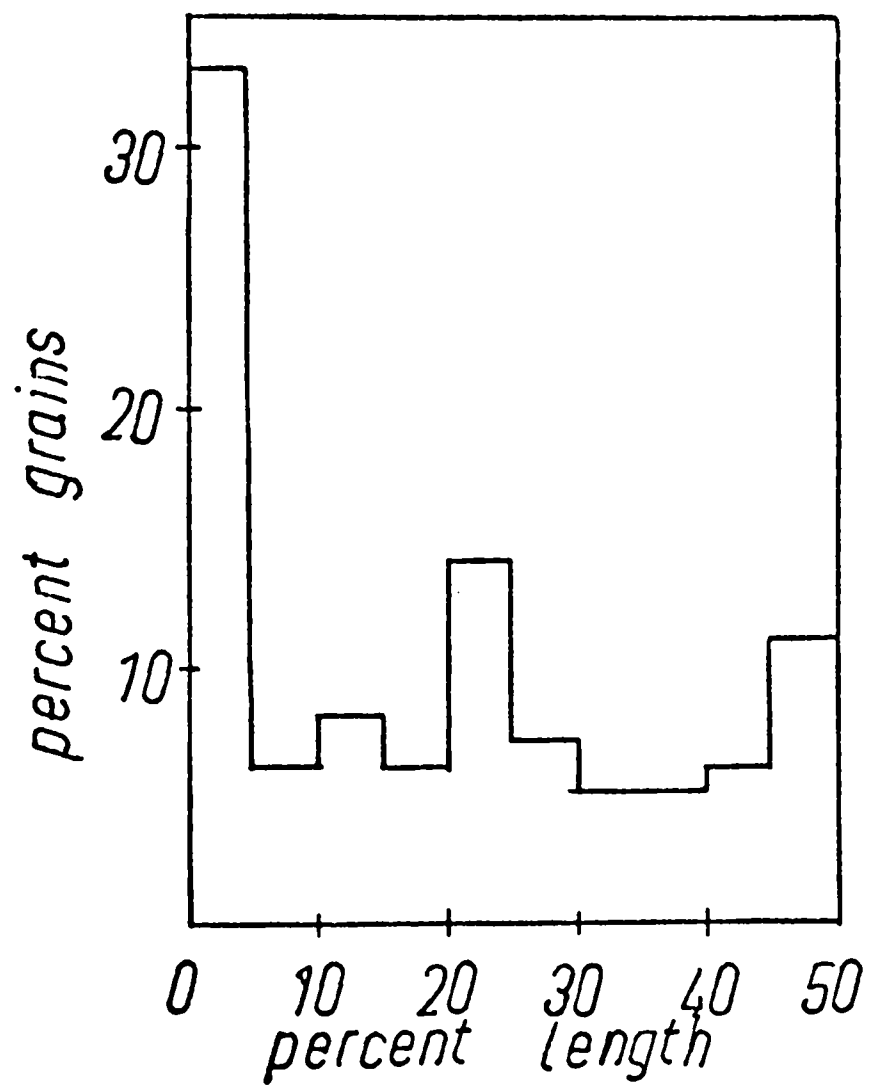
b



19b) 15 min. after removal of cephalixin.

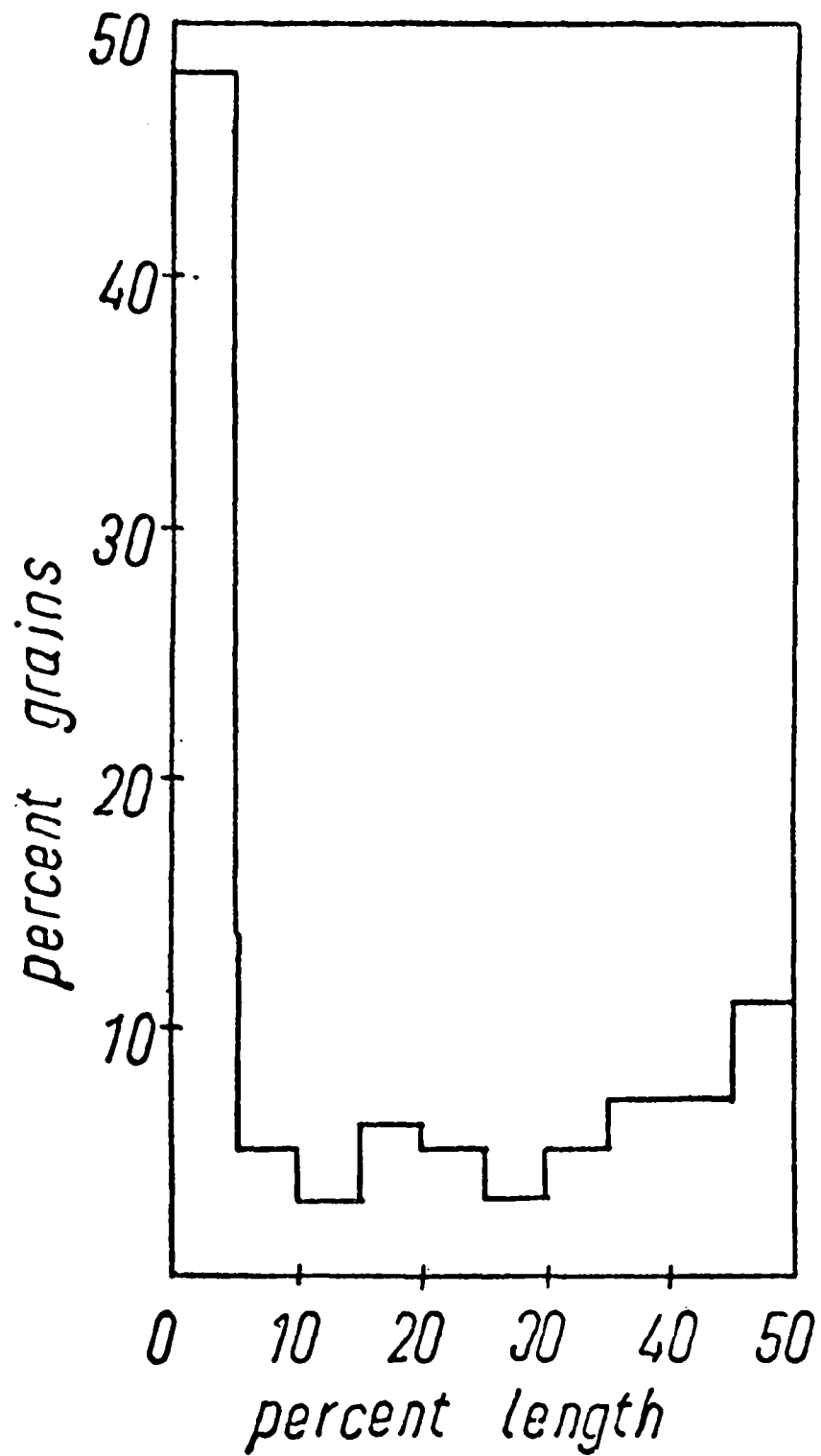
FIGURE 19

c



19c) 30 min. after removal of cephalixin.

FIGURE 20



Distribution of grains (460 grains measured) in cell ghosts of *E.coli*. P530H. Cells were treated with nalidixic acid for one mass doubling and pulse labelled with (3H) histidine for 10 min. in the presence of nalidixic acid immediately before ghost preparation.

the production of PBP3, Spratt (115) has shown that PBP3 is specifically required for cell division in E.coli.

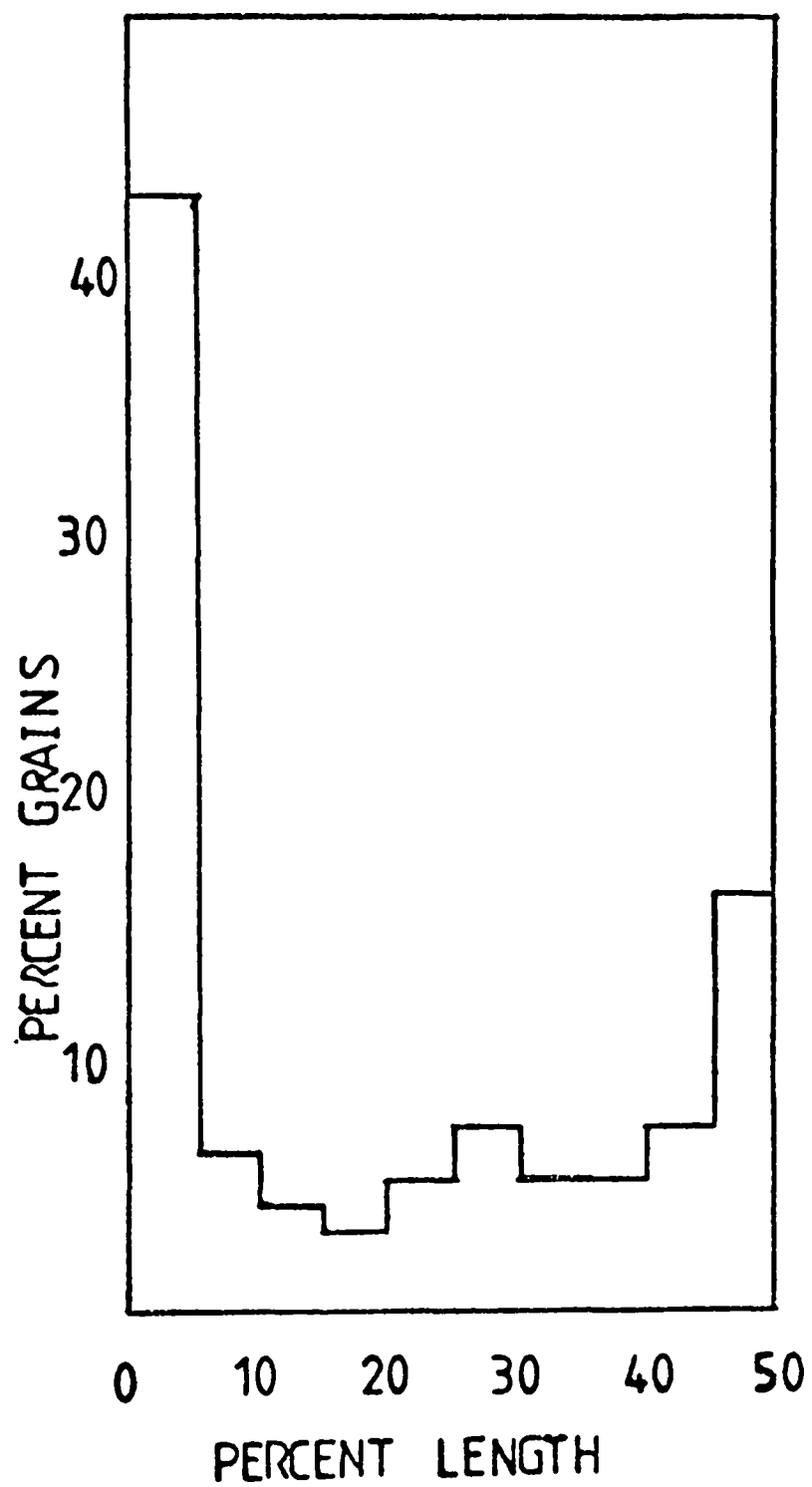
One of these temperature sensitive mutants was obtained and, utilising the fact that the temperature sensitive mutation is co-transducible with leu, (B. Spratt, personal communication), the mutation was transferred to strain P530H. It was hoped that now it would be possible to simulate the conditions obtained through the use of cephalixin by raising the temperature of the new strain (P530HZ). It was thought that pulse labelling at the restrictive temperature might show random distribution of the II* fragment whilst pulse labelling after a short return to the permissive temperature might show a return to the polar/septal concentration pattern thereby establishing a possible connection between PBP3 and the location of protein II*. This was not the case however and Figure 21 shows that the grain distribution obtained by pulse labelling filaments of P530HZ at 44°C is not the same as the random pattern obtained when labelling the presence of cephalixin. Strong polar concentration of labelled protein is apparent.

Data Appraisal

Clearly there are certain objections to reaching immediate, firm conclusions from the data presented above. The preparation of Henning's ghosts involves fairly harsh treatment of the cells, involving as it does detergent treatment followed by urea/trypsin treatment. The major protein being studied is only present in the ghosts as a tryptic fragment. This protein is not the only labelled protein present in the ghosts.

Where possible, an attempt has been made to eliminate

FIGURE 21



Distribution of grains (474 grains measured) on cell ghosts of E.coli. P530HZ. Cells were grown at 44°C. to induce filamentation, for one mass doubling. Cells were then pulse labelled with (3H) histidine for 10 min. at 44°C. before ghost preparation.

artefactual reasons for the labelling pattern observed.

1) For a variety of reasons (including lysis, poor staining etc) many of the ghosts prepared in this work could not be used. Therefore it was necessary to search for and select ghosts which could be seen clearly and which had grains on them which could also be seen clearly and measured accurately. To eliminate the possibility of any personal (unconscious) bias having been exercised, or indeed the possibility that one section of the cell population formed better ghosts than another, The distribution of grains in the ghosts used in various experiments were compared with the expected Poisson distribution. An example of such a check is shown in Figure 22. The cell ghosts fitted a Poisson distribution well in all such tests indicating that the selected ghosts were a fair representation of the general population.

2) Measurements were kept within the limits of resolution of the light microscope. The limit of resolution is usually defined as the minimum distance existing between two points which still permits resolution as two points. In practice this distance approximates to half the wave length of the light source used, a value of about $250 \text{ m}\mu$ - perhaps $240 \text{ m}\mu$ with the best optics and a green filter. This allows cells down to $2.5 \text{ }\mu\text{m}$ in length to be divided into 10% sections, and cells down to $5 \text{ }\mu\text{m}$ in length to be apportioned in 5% sections. Cell ghost lengths in the experiments were well above these basic limits.

3) The possibility that a concentration of labelled protein was observed at the cell poles because labelled protein was being lost from other areas during ghost preparation was considered. This possibility was held to be unlikely in view

Figure 22

Poisson Analysis of Cell Ghosts Described in Figure 20

Number of Grains	0	1	2	3	4	5
Number of Ghosts in each class	14	21	32	21	9	3
Probability	0.137	0.273	0.271	0.180	0.090	0.040
Frequency:-	14	27	27	18	9	4
Observed:-	14	21	32	21	9	4
Difference	0	-6	+5	+3	(0	0)
χ^2	$= \frac{0^2}{14} + \frac{(-6)^2}{27} + \frac{5^2}{27} + \frac{3^2}{18} + \frac{0^2}{13}$					
	$= 0 + 1.33 + 0.93 + 0.5 + 0$					
	$= 2.76 \text{ with four degrees of freedom}$					

This indicates a probability of >0.5 .

∴ The ghosts show a good fit to a Poisson distribution.

of the fact that the presence of cephalixin during the labelling period ensured a random distribution of labelled protein and there seemed to be no conceivable way in which cephalixin could influence matters during ghost preparation. The possibility however was tested in the following fashion.

If the possibility were true, ghosts displaying a random distribution of labelled protein (i.e. labelled in the presence of cephalixin) should contain more labelled protein than ghost showing the pronounced polar label pattern. The experiment described in Figure 19 provided ideal conditions to test this hypothesis. The ghosts used for Figure 19a were prepared 0 min after removal of cephalixin and display a random distribution of label. The ghosts used for Figures 19b and 19c were prepared 15 and 30 min after removal of cephalixin respectively and show non-random distributions of labelled protein. As the autoradiograms for all three preparations were exposed for precisely the same time, the grains per cell value provides a realistic estimate of the relative amounts of protein present in ghosts from all three preparations. There is no significant difference in the grains per cell value for the 0 min sample which shows random label and the 15 and 30 min samples which show strong polar label.

Grains per cell values are 0 min 3.2, 15 min 2.9, 30 min 2.5 (division restarting around this time).

From the grains per cell value and the distribution of the grains as a function of cell length, shown in Figures 19a, b and c, it is possible to calculate a grains per pole value (the pole in this instance being defined as the first 5% of length). The 0 min sample has 0.35 grains per pole,

the 15 min sample 0.81 grains per pole, and the 30 min sample 0.83 grains per pole.

This suggests that the appearance of the polar label pattern after removal of cephalixin is due to an increase in labelled protein at the poles and not to a relative increase at the poles due to spurious loss of labelled protein from other areas.

The conclusion drawn from the above experiments are as follows:

- 1) Protein II* is not distributed evenly over the cell surface, but is concentrated at the cell poles, and possibly at incipient septa.
- 2) Cephalixin prevents this localisation but permits incorporation of protein II* at random sites over the cell surface.
- 3) Subsequent to the removal of cephalixin protein II* is relocated in polar areas.

There was some temptation to interpret the available data in more detail but the conclusions so reached must be considered no more than tentative. There seemed to be no correlation between labelling pattern (.e.g whether or not there is an area of label in the cell centre) and cell length. There also was no evidence that the two poles were differentially labelled.

An alternative experimental approach was now designed to test this conclusion and at the same time explore the possibility that the observed pattern was an artefact only found in mutants which lacked matrix proteins. Bacteriophage K3 uses protein II* as the protein part of its receptor on the surface of E.coli (57). It seemed possible that K3 could be used to 'label' protein II* and thereby reveal any

concentration of the protein at specific sites on the cell surface. In practice preliminary manipulations revealed that K3 had the capacity to "lyse from without", an attribute normally only associated with 'T' phages. K3 phage can 'clear' a logarithmic phase culture containing $\sim 10^8$ cells per ml in three minutes when added at the chosen "labelling"

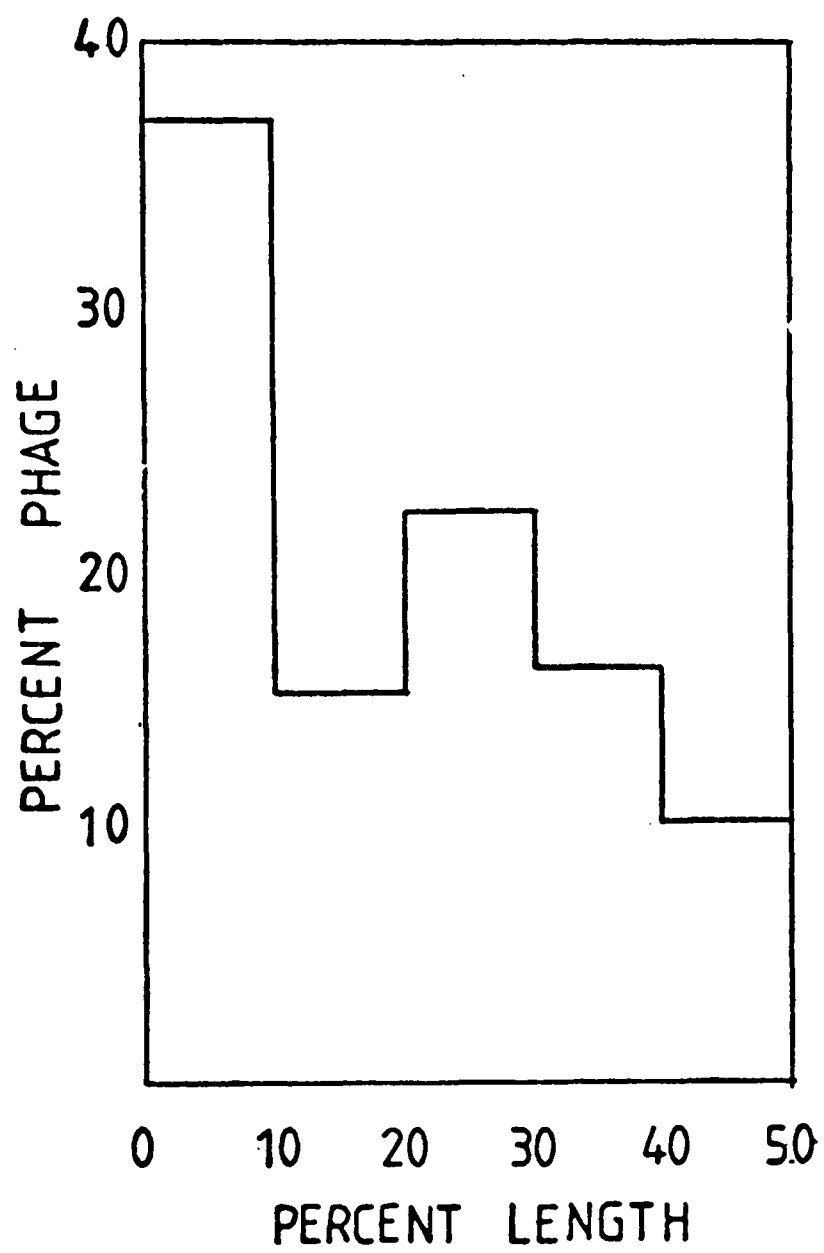
Therefore cells were labelled for only two minutes. concentration of 200 phages per cell./ This was followed by immediate fixation with glutaraldehyde. This system permitted cell survival (in morphological terms) with an average of between twenty and fifty phage adsorbed per cell. Phage position in relation to cell length was measured in electron micrographs.

This type of investigation was made on cells of the three strains used in section C, i.e. P400 the wild type, P530H lacking the matrix proteins, and P530HK3 lacking the matrix proteins and protein II*.

Strain P530HK3 was included as a negative control and did not in fact adsorb any phage at all.

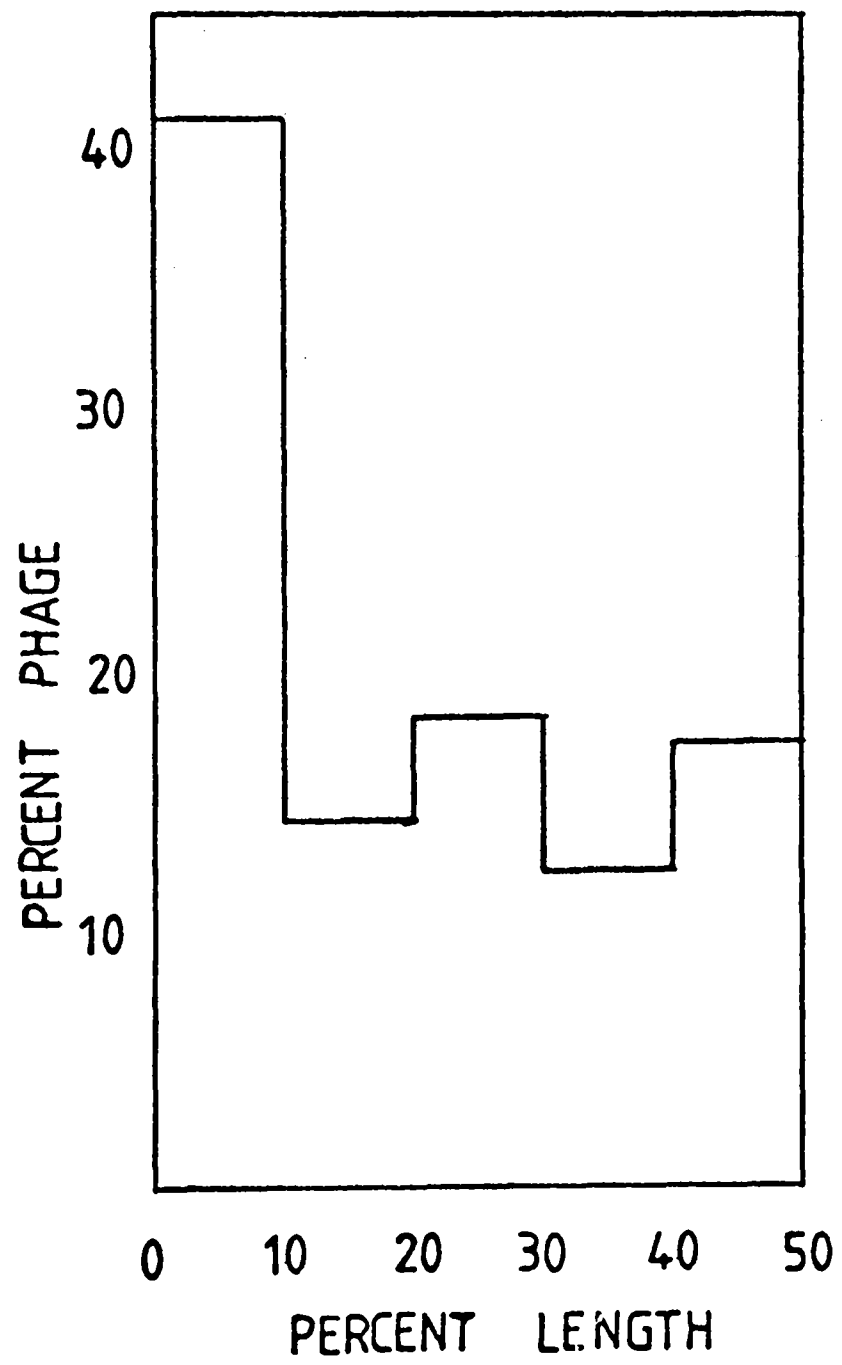
An analysis of the phage distribution on cells of P400 and P530H suggests that, although the receptor sites are not confined to the polar areas, there is a predominance of phage K3 adsorbed at the poles, in the wild type as well as the mutant which lacks matrix proteins (see Figs. 23,24). Although the electron microscopic investigations are only preliminary, it is believed that the results obtained support the conclusions reached from the autoradiographic studies.

FIGURE 23



Distribution of bacteriophage K3 on the surface of cells
of E.coli. P530H after a two minute adsorption period.
(720 phage measured).

FIGURE 24



Distribution of bacteriophage K3 on the surface of cells of E.coli. P400 after a two minute adsorption period. (560 phage measured).

Discussion

The finding that a major outer membrane protein is not evenly distributed over the cell surface poses both problems and questions. The problems arise in the interpretation of labelling experiments. The tendency to interpret areas of dense label (in labelling studies) as being sites of insertion or "growth zones" may not be valid per se. It could be that such areas are areas where the labelled component is concentrated after random insertion.

The fact that the labelling pattern observed after a short pulse is the same as the pattern shown by ghosts obtained from cells grown for up to two generations after pulse labelling, is puzzling. The simple interpretation of the data would suggest that protein II* is continually inserted into the poles, and once inserted, remains there. This seems a highly unlikely situation leading to incredibly dense poles. An alternative hypothesis might be that, although concentrated at the poles, protein II* might migrate to septal sites some time after insertion.

Why protein II* should be present in concentrated amounts at the cell poles remains unknown. The cell poles, of course, stand out as being different in terms of shape, and being the areas where cell envelope components must "turn corners". Cytochemical studies have revealed high polar concentration of certain phosphatases in E.coli (141) and more recently Dietzel et al (142) have found that the induction of maltose binding protein results in the formation of large "polar caps". These caps are a result of enlargement of the periplasmic space and the authors propose that this is the result of maltose binding protein being concentrated at

the cell poles. It has been proposed that the cell poles are the sites of new envelope growth in E.coli (143,103,104) and this has been shown to be so, in the case of the rod-shaped fission yeast Schizosaccharomyces pombe (144), and in Bdellovibrio bacteriovorus (145). There are therefore, ample grounds for believing the cell poles to be "special", however, no definite proposals can be made for the role of protein II* at these areas. A role in the maintenance of cell shape seems most likely in view of the findings of Sonntag et al (36) in their studies on mutants lacking protein II* and lipoprotein.

The questions surrounding the role of outer membrane proteins in cell shape and architecture remain intriguing. It was proposed by Garten and Henning (146) and Haller and Henning (147) that protein II* and the matrix proteins (Ia and Ib) played an important part in determining cell shape. This view had to be modified when stable rod-shaped mutants which lacked these proteins were discovered (128), however, the recent finding that mutants which lacked protein II* and lipoprotein did in fact lose rigidity and shape (36) has renewed interest in the role of these proteins in the determination of cell shape.

Taking a broad general view of the information currently available on components of the outer membrane of E.coli, it seems reasonable to propose the following. The outer membrane consists of a lipid bilayer. The phospholipid forms the inner aspect of the bilayer, and the lipid portion of the lipopolysaccharide forms the outer one. The proteins of the outer membrane are not all evenly distributed over the membrane and protein interactions could be responsible for differential concentrations and varying structure within

the membrane. Information about membrane protein interactions is currently becoming available and it is known that 1) matrix protein is specifically cross linked to itself when whole cells are treated with cleavable azide reagents (148), 2) matrix protein appears to exist as a trimer in the outer membrane (149) and 3) protein II* and lipoprotein can be crosslinked to each other and to peptidoglycan (148).

Future Studies

It seems likely that the autoradiographic technique has yielded as much information as is possible within its limitations. The greater resolving power of the electron microscope is required if a detailed study of the insertion and mobility of protein II* is to be pursued. Perhaps the isolation of a K3 amber mutation in a strain carrying a temperature sensitive amber suppressor would provide a convenient "switching mechanism" to study the insertion of K3 receptor sites, and therefore protein II*. This type of study could be achieved either with a phage label or with ferritin labelled antibody to protein II*. An examination of the relative amounts of outer membrane proteins in mini-cells versus filaments in a mini-cell producing strain of E.coli might provide information about asymmetric distribution of these proteins. This kind of investigation has been undertaken previously (150), but before modern resolution of the outer membrane proteins.

Declaration

None of the material in this thesis has been previously submitted for a degree to any University or Institution. All the experiments were designed and executed by myself. Early polyacrylamide gel analysis (referred to but not shown) was performed by Dr. J F Lutkenhaus and Mrs L Richardson. Experimental results and implications were discussed frequently with my supervisor Dr W D Donachie. Part of the experimental studies section has been published. A copy of the paper is bound with the text.

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Literature Cited

1. Cairns, J. (1963) The chromosome of Escherichia coli. Cold Spring Harbor Symposia on Quantitative Biology 28, 43-45
2. Masters, M. and P. Broda (1971) Evidence for the bi-directional replication of the E.coli chromosome. Nature New Biol. (London) 232, 137-140
3. Donachie, W.D. (1968) Relationship between cell size and time of initiation of DNA replication. Nature, London 219, 1077-1079
4. Donachie, W.D. and M. Masters (1969) Temporal control of gene expression in bacteria. The Cell Cycle, eds. G.M. Padilla, G.L. Whitson and J.L. Cameron, pp. 37-76 New York: Academic Press
5. Helmstetter, C.E. (1967) Rate of DNA synthesis during the division cycle of Escherichia coli B/r. J.Mol.Biol. 24, 417-427
6. Clark, D.J. (1968) The regulation of DNA replication and cell division in Escherichia coli B/r. Cold Spring Harbor Symposia on Quantitative Biology. 33, 823-838
7. Helmstetter, C.E. and O. Pierucci (1968) Cell division during inhibition of DNA synthesis in Escherichia coli. J.Bacteriol. 95, 1627-1633
8. Jones, N.C. and W.D. Donachie (1973) Chromosome replication, transcription and control of cell division in Escherichia coli. Nature New Biol. (London) 243, 100-103
9. Worcel, A. and E. Burgi (1974) Properties of a membrane attached from the folded chromosome of E.coli. J.Mol. Biol. 82, 91-105
10. Pettijohn, D.E., R.M. Hecht, G.O. Stonington and T.D. Stamato (1973) Steenbock symposium on "DNA synthesis in vitro". p. 145. University Park Press, London
11. Jones, N.C. and W.D. Donachie (1974) Protein synthesis and the release of the replicated chromosome from the cell membrane. Nature New Biol. (London) 251, 252-254
12. Nicolaidis, A.A. and I.B. Holland (1978) Evidence for the specific association of the chromosomal origin with outer membrane fractions isolated from Escherichia coli. J.Bacteriol. 135, 178-189
13. Kjedelgaard, N.O., O. Maaloe and M. Schaechter (1958) The transition between different physiological states during balanced growth of Salmonella typhimurium. J.gen. Microbiol. 19, 607-616
14. Osborn, M.J., J.E. Gander, E. Parisi and J. Carson (1972) Mechanism of assembly of the outer membrane of Salmonella typhimurium. J.Biol.Chem. 247, 3962-3972

15. Mitchell, P. and J. Moyle (1956) Osmotic function and structure in bacteria. in Bacterial Anatomy. eds. E.T.C. Spooner and B.A.D. Stocker, University Press, Cambridge
16. Miura, T. and S. Mizushima (1969) Separation and properties of outer and cytoplasmic membranes in Escherichia coli. Biochim.Biophys.Acta. 193, 268-278
17. Mizushima, S. and H. Yamada (1975) Isolation and characterisation of two outer membranes from Escherichia coli. Biochim.Biophys.Acta. 375, 44-53
18. Schnaitman, C.A. (1970) Examination of the protein composition of the cell envelope of Escherichia coli by polyacrylamide gel electrophoresis. J.Bacteriol. 104, 882-889
19. Martin, E.L. and R.A. MacLeod (1971) Isolation and chemical composition of the cytoplasmic membrane of a Gram negative bacterium. J.Bacteriol. 105, 1160-1167
20. Singer, S.J. and G.L. Nicolson (1972) The fluid mosaic model of the structure of cell membranes. Science, 175, 720-731
21. Fox, C.F. (1972) The structure of cell membranes. Sci.Amer. 226, 30-38
22. Rogers, H.J. (1970) Bacterial growth and the cell envelope. Bacteriol.Rev. 34, 194-204
23. Hinckley, A., E. Müller and L. Rothfield (1972) Reassembly of a membrane-bound multienzyme system. I. Formation of a particle containing phosphatidylethanolamine, lipopolysaccharide and two glycosyltransferase enzymes. J.Biol.Chem. 247, 2623-2628
24. Weidel, H. and H. Pelzer (1964) Bagshaped macromolecules - a new outlook on bacterial cell walls. Adv.Enzymol. 26, 193-232
25. Ghuysen, J.M. (1968) Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bacteriol.Rev. 32, 425-464
26. Verwer, R.W.H., N. Nanninga, W. Keck and U. Schwarz (1978) Arrangement of glycan chains in the sacculus of Escherichia coli. J.Bacteriol. 136, 723-729
27. Marr, A.G., R.J. Harvey and W.C. Trentini (1966) Growth and division of Escherichia coli. J.Bacteriol. 91, 2388-2389
28. Henning, U., K. Rehn, and B. Hoehn (1973) Cell envelope and shape of Escherichia coli K-12. Proc.Natl.Acad.Sci. USA 70, 2033-2036
29. Henning, U. and U. Schwarz (1973) In Bacterial Membranes and Walls. ed. L. Leive 413-438. New York, Dekker.

30. Ryter, A., Y. Hirota and U. Schwarz (1973) Process of cellular division in Escherichia coli. Growth pattern of E.coli murein. J.Mol.Biol. 78, 185-195
31. Braun, V and K. Rehn (1969) Chemical characterisation, spatial distribution and function of a lipoprotein (murein lipoprotein) of the E.coli cell wall. Eur.J. Biochem. 10, 426-438
32. Hantke, K. and V. Braun (1973) Covalent binding of lipid to protein. Diglyceride and amid-linked fatty acid at the N-terminal end of the murein lipoprotein of the Escherichia coli outer membrane. Eur.J.Biochem. 34, 284-296
33. Inouye, M., J. Shaw and C. Shen (1972) The assembly of a structural lipoprotein in the envelope of Escherichia coli. J.Biol.Chem. 247, 8154-8159
34. Braun, V. and V. Bosch (1972) Repetitive sequences in the murein-lipoprotein of the cell wall of Escherichia coli. Proc.Natl.Acad.Sci.USA 69, 970-974
35. Hirota, Y., H. Suzuki, Y. Nishimura and S. Yasuda (1977) On the process of cellular division in Escherichia coli: A mutant of E.coli lacking a murein-lipoprotein. Proc. Natl.Acad.Sci.USA 74, 1417-1420
36. Sonntag, I., H. Schwarz, Y. Hirota and U. Henning (1978) Cell envelope and shape of Escherichia coli. Multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J.Bacteriol. 136, 280-285
37. Smit, J., Y. Kamio and H. Nikaido (1975) Outer membrane of Salmonella typhimurium: Chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. J.Bacteriol. 124, 942-958
38. Schnaitman, C. (1974) Outer membrane proteins of Escherichia coli III. Evidence that the major protein of Escherichia coli 0111 outer membrane consists of four distinct polypeptide species. J.Bacteriol. 118, 442-451
39. Henning, U., B. Høhn and I. Sonntag (1973) Cell envelope and shape of E.coli K-12. The ghost membrane. Eur.J.Biochem. 39, 27-36
40. Schmitges, C.J. and U. Henning (1976) The major proteins of the E.coli outer cell envelope membrane. Heterogeneity of protein I. Eur.J.Biochem. 63, 47-52
41. Lugtenberg, C., J. Meijers, R. Peters, P. van der Hoeck, and L. van Alphen (1975) Electrophoretic resolution of the major outer membrane protein of Escherichia coli K-12 into four bands. FEBS Lett. 58, 254-258
42. Nakamura, K. and S. Mizushima (1976) Effects of heating in dodecylsulphate on the conformation and electrophoretic mobility of isolated major outer membrane proteins from Escherichia coli K-12. J.Biochem. (Tokyo) 80, 1411-1422

43. Rosenbusch, J.P. (1974) Characterisation of the major envelope protein from Escherichia coli. Regular arrangement on the peptidoglycan and unusual dodecyl sulphate binding. J.Biol.Chem. 249, 8019-8029
44. McMichael, J.C. and J.T. Ou (1977) Metal ion dependence of a heat modifiable protein from the outer membrane of Escherichia coli upon sodium dodecyl sulphate gel electrophoresis. J.Bacteriol. 132, 314-320
45. Braun, V., H. Gnirke, U. Henning and K. Rehn (1973) Model for the structure of the shape maintaining layer of the Escherichia coli cell envelope. J.Bacteriol. 114, 1264-1270
46. Nakae, T. (1976) Identification of the outer membrane protein of E.coli that produces transmembrane channels in reconstituted vesicle membranes. Biochem.Biophys.Res. Commun. 71, 877-884
47. Lutkenhaus, J.F. (1977) Role of a major outer membrane protein in Escherichia coli. J.Bacteriol. 131, 631-637
48. Datta, D.B., B. Arden and U. Henning (1977) Major proteins of the Escherichia coli outer cell envelope membrane as bacteriophage receptors. J.Bacteriol. 131, 821-829
49. Hantke, K. (1978) Major outer membrane proteins of E.coli K-12 serve as receptors for the phages T2 (protein Ia) and 434 (protein Ib). Molec.Gen.Genet. 164, 131-134
50. Lugtenberg, B., R. Peters, H. Bernheimer and W. Berendsen (1976) Influence of cultural conditions and mutations on the composition of the outer membrane proteins of Escherichia coli. Molec.Gen.Genet. 147, 251-262
51. Hindennach, L. and U. Henning (1975) The major proteins of the Escherichia coli outer cell envelope membrane. Eur.J.Biochem. 59, 207-213
52. Di Rienzo, J.M., K. Nakamura and M. Inouye (1978) The outer membrane proteins of Gram negative bacteria: Biosynthesis assembly and functions. Ann.Rev.Biochem. 47, 481-532
53. Alphen, L. van, B. Lugtenberg, R. van Boxtel, A. Hack, C. Verhoef and L. Havekes (1979) meoA is the structural gene for outer membrane protein C of Escherichia coli K-12. Molec.Gen.Genet. 169, 147-155
54. Ichihara, S., S. Mizushima (1978) Characterisation of major outer membrane proteins O8 and O9 of Escherichia coli K-12. Evidence that structural genes for the two proteins are different. J.Biochem. (Tokyo) 83, 1095-1100
55. Verhoef, C. B. Lugtenberg, R. van Boxtel, P. de Graaff and H. Verheij (1979) Genetics and biochemistry of the peptidoglycan association proteins b and c of Escherichia coli K-12. Molec.Gen.Genet. 169, 137-146

56. Endermann, R., C. Krämer and U. Henning (1978) Major outer membrane proteins of Escherichia coli K-12. Evidence for protein II* being a transmembrane protein. FEBS Lett. 86, 21-24
57. Alphen, L. van, L. Havekes and B. Lugtenberg (1977) Major outer membrane d of Escherichia coli K-12: purification and in vitro activity of bacteriophage K3 and F-pilus mediated conjugation. FEBS Lett. 75, 285-290
58. Skurray, R.A., R.E.W. Hancock and P. Reeves (1974) Con⁻ mutants: class of mutants in Escherichia coli K-12 lacking a major cell wall protein and defective in conjugation and adsorption of a bacteriophage. J.Bacteriol. 119, 726-735
59. Manning, P.A. and P. Reeves (1976) Outer membrane proteins of Escherichia coli K-12: differentiation of proteins 3a and 3b on acrylamide gels and further characterisation of con (tolG) mutants. J.Bacteriol. 127, 1070-1079
60. Datta, D.B., C Krämer and U. Henning (1976) Diploidy for a structural gene specifying a major protein of the outer cell envelope membrane of Escherichia coli K-12. J.Bacteriol. 128, 834-841
61. Bachmann, B.J., K.B. Low and A.L. Taylor (1976) Recalibrated linkage map of Escherichia coli K-12. Bact.Rev. 40, 116-167
62. Inouye, S., N. Lee, M. Inouye, H.C. Wu, H. Suzuki, Y. Nishimura, H. Iketani and Y. Hirota (1977) Amino acid replacement of a mutant lipoprotein of the Escherichia coli outer membrane. J.Bacteriol. 132, 308-313
63. Lee, D.L., C.A. Schnaitman and A.P. Pugsley (1979) Chemical heterogeneity of major outer membrane pore proteins of Escherichia coli. J.Bacteriol. 138, 861-870
64. Inouye, S., S. Wang, J. Sekizawa, S. Halegoua and M. Inouye (1977) Amino acid sequence for the peptide extension on the prolipoprotein of the Escherichia coli outer membrane. Proc.Natl.Acad.Sci.USA 74, 1004-1008
65. Blobel, G. and B. Dobberstein (1975) Transfer of proteins across membranes. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane bound ribosomes of murine myeloma. J.Cell.Biol. 67, 835-851
66. Silhavy, T.J., M.J. Casadaban, H.A. Shuman and J.R. Beckwith (1976) Conversion of β -galactosidase to a membrane bound state by gene fusion. Proc.Natl.Acad.Sci. USA 73, 3423-3427

67. Henning, U., W. Schmidmayr and I. Hindennach (1977) Major proteins of the outer cell envelope membrane of Escherichia coli K-12: multiple species of protein I. Mol.Gen.Genet. 154, 293-298
68. Alphen, W. van, N. van Selm and B. Lugtenberg (1978) Involvement of proteins b and e in the functioning of pores for nucleotides. Mol.Gen.Genet. 159, 75-83
69. Foulds, J. and T. Chai (1978) A new major outer membrane protein found in an Escherichia coli tolF mutant resistant to phage Tu 1b. J.Bacteriol. 133, 1478-1485
70. Foulds, J. and T. Chai (1978) Chromosomal location of a gene (nmpA) involved in expression of a major outer membrane protein in Escherichia coli. J.Bacteriol. 136, 501-506
71. Chai, T. and J. Foulds (1979) Inactivation of bacteriophages by protein E, a new major membrane protein isolated from an Escherichia coli mutant. J.Bacteriol. 137, 226-233
72. Lin, E., Y. Hirota and F. Jacob (1971) On the process of cellular division in Escherichia coli. VI. Use of a methocell-autoradiographic method for the study of cellular division in Escherichia coli. J.Bacteriol. 108, 375-385
73. Green, E. and M. Schaechter (1972) The mode of segregation of the bacterial cell membrane. Proc.Natl.Acad.Sci.USA 69, 2312-2316
74. Overath, P., F.F. Hill and I. Lamnek (1971) Biogenesis of Escherichia coli membranes: evidence for randomisation of the lipid phase. Nature New Biol. 234, 264-267
75. Morrison, D.A. and H.J. Morowitz (1970) Studies on membrane synthesis in Bacillus megaterium KM. J.Mol.Biol. 49, 441-
76. Cronan, J.E. and P.R. Vagelos (1972) Metabolism and function of the membrane phospholipids of Escherichia coli. Biochim.Biophys.Acta. 265, 25-60
77. Hawrot, E. and E.P. Kennedy (1975) Biogenesis of membrane lipids: mutants of Escherichia coli with temperature-sensitive phosphatidylserine decarboxylase. Proc.Natl.Acad.Sci.USA 72, 1112-1116
78. Autissier, F. (1971) PhD thesis, University of Paris. Contribution a l'etude du mode de croissance des membranes bacteriennes. Segregation de marqueurs membranaires chez Escherichia coli.
79. Tsukagashi, N., P. Fielding and C. Fox (1971) Membrane assembly in Escherichia coli. I. Segregation of preformed and newly formed membrane into daughter cells. Biochem.Biophys.Res.Comm. 44, 497-502

80. Wilson, G. and C. Fox (1971) Biogenesis of microbial transport systems: Evidence for coupled incorporation of newly synthesised lipids and proteins into the membrane. *J.Mol.Biol.* 55, 49-60
81. Pierucci, O. (1979) Phospholipid synthesis during the cell division cycle of Escherichia coli. *J.Bacteriol.* 138, 453-460
82. Hoffman, B., W. Messer and U. Schwarz (1972) Regulation of polar cap formation in the life cycle of Escherichia coli. *J.Supramol.Struct.* 1, 29-37
83. Hakenbeck, R. and W. Messer (1977) Oscillations in the synthesis of cell wall components in synchronised cultures of Escherichia coli. *J.Bacteriol.* 129, 1234-1238
84. Churchward, G.G. and J.B. Holland (1976) Envelope synthesis during the cell cycle in Escherichia coli B/r. *J.Mol.Biol.* 105, 245-261
85. Donachie, W.D., K.J. Begg and M. Vicente (1976) Cell length, cell growth and cell division. *Nature (London)* 264, 328-333
86. Beachey, E. and R. Cole (1966) Cell wall replication in Escherichia coli, studied by immunofluorescence and immunoelectron microscopy. *J.Bacteriol.* 92, 1245-1251
87. Grant, W.D., I.W. Sutherland and J.F. Wilkinson (1969) Exopolysaccharide colanic acid and its occurrence in the Enterobacteriaceae. *J.Bacteriol.* 100, 1187-1193
88. Higgins, M. and G. Shockman (1971) Prokaryotic cell division with respect to wall and membranes. *CRC Crit. Rev.Microbiol.* 1, 29-72
89. Briles, E. and A. Tomasz (1970) Radioautographic evidence for equatorial wall growth in a Gram positive bacterium. *J.Cell Biol.* 47, 786-790
90. Autissier, F., A. Haffe and A. Kepes (1971) Segregation of galactoside permease, a membrane marker during growth and cell division in Escherichia coli. *Mol.Gen.Genet.* 112, 275-288
91. Autissier, F. (1972) NATO symposium on DNA replication and the cell membrane. Cortina-de-Ampezzo, Italy.
92. Tubergen, R.P. van, and R.B. Setlow (1961) Quantitative radioautographic studies on exponentially growing cultures of Escherichia coli. *Biophys.J.* 1, 589-625
93. Schwarz, U., A. Ryter, A. Rambach and Y. Hirota (1975) Process of cellular division in Escherichia coli. Differentiation of growth zones in the sacculus. *J.Mol. Biol.* 98, 749-759

94. Daneo-Moore, L. and G.D. Shockman (1977) The bacterial cell surface in growth and division. p. 597-715, in Cell surface reviews (ed. G.D. Poste and G. Nicolson), vol. 4. Elsevier/North Holland Publishing Co, Amsterdam.
95. James, R., J.Y. Haga and A.B. Pardee (1975) Inhibition of an early event in the cell division cycle of Escherichia coli by FL1060, an amidinopenicillanic acid. J.Bacteriol. 122, 1283-1292
96. Spratt, B.G. (1975) Distinct penicillin-binding proteins involved in the division elongation and shape of Escherichia coli. Proc.Natl.Acad.Sci.USA 72, 2999-3003
97. Spratt, B.G. (1977) Temperature-sensitive cell division mutants of Escherichia coli with thermolabile penicillin-binding proteins. J.Bacteriol. 131, 293-305
98. Iwaya, M., R. Goldman, D.J. Tipper, B. Feingold and J.L. Strominger (1978) Morphology of an Escherichia coli mutant with a temperature-dependent round cell shape. J.Bacteriol. 136, 1143-1158
99. Satta, G., R. Fontana, P. Canepari and G. Botta (1979) Peptidoglycan synthesis in cocci and rods of a pH-dependent morphologically conditional mutant of Klebsiella pneumoniae. J.Bacteriol. 137, 727-734
100. Jacob, F., S. Brenner and F. Cuzin (1963) On the regulation of DNA replication in bacteria. Cold Spring Harbor Symposia on Quantitative Biology. 28, 329-348
101. Donachie, W.D. and K.J. Begg (1970) Growth of the bacterial cell. Nature (London) 227, 1220-1224
102. Leal, J. and H. Marcovich (1971) Segregation of T6 phage receptors during cell division in Escherichia coli K-12. Ann.Inst.Pasteur 120, 467-474
103. Begg, K.J. and W.D. Donachie (1973) Topography of outer membrane growth in E.coli. Nature New Biol. 245, 38-39
104. Begg, K.J. and W.D. Donachie (1977) Growth of the cell surface of Escherichia coli. J.Bacteriol. 129, 1524-1536
105. Ryter, A., H. Shuman and M. Schwarz (1975) Integration of the receptor for bacteriophage lambda in the outer membrane of Escherichia coli: coupling with cell division, J.Bacteriol. 122, 295-301
106. Muhlradt, P.F., J. Menzel, J.R. Golecki and V. Speth (1973) Outer membrane of Salmonella: site of export of newly synthesised lipopolysaccharide on the bacterial surface. Eur.J.Biochem. 35, 471-481
107. Muhlradt, P.F., J. Menzel, J.R. Golecki and V. Speth (1974) Lateral mobility and surface density of lipopolysaccharide in the outer membrane of Salmonella typhimurium. Eur.J.Biochem. 43, 533-539

108. Manning, P.A., A. Purspurs and P. Reeves (1976) Outer membrane of Escherichia coli K-12: isolation of mutants with altered protein 3A by using host range mutants of bacteriophage K3. J.Bacteriol. 127, 1080-1084
109. Datta, D.B., B. Arden and U. Henning (1977) Major proteins of the Escherichia coli outer cell envelope membrane as bacteriophage receptors. J.Bacteriol. 131, 821-829
110. Anderson, E.H. (1946) Growth requirements of virus resistant mutants of E.coli strain B. Proc.Natl.Acad. Sci USA 32, 120-128
111. Matzura, H. , S. Molin and O. Maaloe (1971) Sequential biosynthesis of the β and β' subunits of the DNA dependent RNA polymerase from Escherichia coli. J.Mol.Biol. 59, 17-25
112. Helmstetter, C.E. (1967) Rate of DNA synthesis during the division cycle of E.coli B/r. J.Mol.Biol. 24, 417-428
113. Skurray, R.A., R.E.W. Hancock and P. Reeves (1974) Con⁻ mutants: class of mutants in Escherichia coli K-12 lacking a major cell wall protein and defective in conjugation and adsorption of a bacteriophage. J.Bacteriol. 119, 726-735
114. Davies, J.K. and P. Reeves (1975) Genetics of resistance to colicins in Escherichia coli K-12: cross resistance among colicins of group A. J.Bacteriol. 123, 102-117
115. Spratt, B.G. (1977) Temperature-sensitive cell division mutants of Escherichia coli with thermolabile penicillin-binding proteins. J.Bacteriol. 131, 293-305
116. Begg, K.J. and W.D. Donachie (1978) Changes in cell size and shape in thymine-requiring Escherichia coli associated with growth in low concentrations of thymine. J.Bacteriol. 133, 452-458
117. Henning, U., K. Rehn and B. Hoehn (1973). Cell envelope and shape of Escherichia coli K-12. Proc.Natl.Acad.Sci. USA 70, 2033-2036
118. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-685
119. Davis, B.J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. Annals N.Y. Acad. Sci. 121, 404-427
120. Ornstein, L. (1964) Disc electrophoresis. I. Background and theory. Annals N.Y. Acad.Sci. 121, 321-349
121. Studier, F.W. (1973) Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J.Mol.Biol. 79, 237-248

122. Starka, J. (1971) Formation of stabilite osmotique des formes filamenteuses d'Escherichia coli induites par la penicilline. Ann.Inst.Pasteur; Paris 121, 149-159
123. Nishima, T. and S. Nakazawa (1973) Cephalixin induced morphological alterations in the surface structures of S.aureus and E.coli demonstrated by scanning electron microscopy. Jap.J.Microbiol. 17, 383-392
124. Swanstrom, M., M.H. Adams (1951) Agar layer method for the production of high titre phage stocks. Proc.Soc. Exptl.Biol.Med. 78, 372-375
125. Gregory, D.W. and B.J.S. Pirie (1972) Wetting agents for electron microscopy of biological specimens, p. 234-235 in Proceedings of the Fifth European Congress on Electron Microscopy. (ed. A.M. Glavert) Institute of Physics, London.
126. Starka, J. and J. Moravova (1967) Cellular division of penicillin induced filaments of Escherichia coli. Folia Microbiologica 12, 240-245
127. Alphen, W. van, B. Lugtenberg and W. Berendsen (1976) Heptose deficient mutants of Escherichia coli K-12 deficient in up to three major outer membrane proteins. Mol.Gen.Genet. 147, 263-269
128. Henning, U. and I. Haller (1975) Mutants of Escherichia coli K-12 lacking all "major" proteins of the outer cell envelope membrane. FEBS Lett. 55, 161-164
129. Smit, J. and H. Nikaido (1978) Outer membrane of Gram negative bacteria. XVIII Electron microscopic studies on porin insertion sites and growth of cell surface of Salmonella typhimurium. J.Bacteriol. 135, 687-702
130. Bayer, M.E. (1968) Adsorption of bacteriophages to adhesions between wall and membrane of Escherichia coli. J.Virol. 2, 346-356
131. Inouye, M., J. Shaw and C. Shen (1972) The assembly of a structural lipoprotein in the envelope of Escherichia coli. J.Biol.Chem. 247, 8154-8159
132. Weigand, R.A., K.D. Vinci and L.I. Rothfield (1976) Morphogenesis of the bacterial division septum: a new class of septation-defective mutants. Proc.Natl.Acad. Sci.USA 73, 1882-1886
133. Sarma, V. and P. Reeves (1977) Genetic locus (ompB) affecting a major outer membrane protein in Escherichia coli K-12. J.Bacteriol. 132, 23-27
134. Schweizer, M., I. Hindennach and U. Henning (1978) Major proteins of the Escherichia coli outer cell envelope membrane. Interaction of protein II* with lipopoly-saccharide. Eur.J.Biochem. 82, 211-217

135. Muhlradt, P.F. and J.R. Golecki (1975) Asymmetrical distribution and artifactual re-orientation of lipopolysaccharide in the outer membrane bilayer of Salmonella typhimurium. Eur.J.Biochem. 51, 343-352
136. Blumberg, P.M. and J.L. Strominger (1974) Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. Bacteriol.Rev. 38, 291-335
137. Spratt, B.G. (1977) Properties of the penicillin-binding proteins of Escherichia coli K-12. Eur.J.Biochem. 72, 341-352
138. Spratt, B.G. and J.L. Strominger (1976) Identification of the major penicillin-binding proteins of Escherichia coli as D-alanine carboxypeptidase 1A. J.Bacteriol. 127, 660-663
139. Spratt, B.G. (1975) Distinct penicillin-binding proteins involved in the division elongation and shape of Escherichia coli K-12. Proc.Natl.Acad.Sci.USA 72, 2999-3003
140. Spratt, B.G. (1977) Comparison of the binding properties of two 6 β amidinopenicillanic acid derivatives that differ in their physiological effects on Escherichia coli. Antimicrob.Agents Chemother. 11, 161-166
141. Wetzel, B.K., S.S. Spicer, H.F. Dvorak and L.E. Heppel (1970) Cytochemical localisation of certain phosphatases in Escherichia coli. J.Bacteriol. 104, 529-542
142. Diezel, I., V. Kolb and W. Boos (1978) Pole cap formation in Escherichia coli following induction of the maltose binding protein. Arch.Microbiol. 118, 207-218
143. Bisset, K.A. and P.E. Pease (1957) The distribution of Flagella in dividing bacteria. J.Gen.Microbiol. 16, 382-384
144. Streiblova, E. and A. Wolf (1972) Cell wall growth during the cell cycle of Schizosaccharomyces pombe. Z.Allg.Mikrobiol. 12, 673-684
145. Eksztejn, M. and M. Varon (1977) Elongation and cell division in Bdellovibrio bacteriovorus. Arch.Microbiol. 114, 175-181
146. Garten, W. and U. Henning (1974) Cell envelope and shape of Escherichia coli K-12. Isolation and preliminary characterisation of the major ghost membrane proteins. Eur.J.Biochem. 47, 343-352
147. Haller, I. and U. Henning (1974) Cell envelope and shape of Escherichia coli K-12. Crosslinking with dimethyl imidoesters of the whole cell wall. Proc.Natl.Acad.Sci. USA 71, 2018-2021

148. Reithmeier, R.A.F. and P.D. Bragg (1977) Cross-linking of the proteins in the outer membrane of Escherichia coli. Biochim.Biophys.Acta. 466, 245-256
149. Palva, E.T. and L.L. Randall (1978) Arrangement of protein I in Escherichia coli outer membrane: Cross-linking study. J.Bacteriol. 133, 279-286
150. Goodell, E.W., U. Schwarz and R.M. Teather (1974) Cell envelope composition of Escherichia coli K-12: a comparison of the cell poles and the lateral wall. Eur.J.Biochem. 47, 567-572
151. Garten, W., I. Hindennach and U. Henning (1975) The major proteins of the Escherichia coli outer cell envelope membrane. Characterisation of proteins II* and III, comparison of all proteins. Eur.J.Biochem. 59, 215-221
152. Weidel, W., H. Frank and H.H. Martin (1960) The rigid layer of the cell wall of Escherichia coli strain B. J.Gen.Microbiol. 22, 158-166

Cell Surface Growth in *Escherichia coli*: Distribution of Matrix Protein

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Autoradiography of cell envelope "ghosts" from *Escherichia coli* was used to demonstrate that newly synthesized molecules of "matrix" protein are inserted at random locations over the entire surface of the outer membrane and that, once inserted, these molecules are not thereafter conserved in any fixed spatial location.

The cell envelope of *Escherichia coli* is a complex structure which is basically composed of three distinct layers: (i) the cytoplasmic membrane consisting of proteins and phospholipids, (ii) the peptidoglycan layer, and (iii) an outer membrane containing proteins, lipopolysaccharide, and phospholipids (15). Many growth studies have been carried out on the cell envelope; nevertheless, contradictory conclusions have been drawn from these studies. The evidence in this field can be divided into two broadly based contradictory groups: (i) the evidence that supports the view that cell wall growth is an intercalary process which allows components to diffuse into the wall at random (3, 8, 12, 18), and (ii) the evidence which forms the tenet that envelope growth involves localized insertion of new material into a few "growth zones" per cell (4, 5, 7, 11).

Rosenbusch (16) has described a technique which produces rod-shaped cell "ghosts" containing the peptidoglycan layer of the cell envelope surrounded by one major outer membrane protein (matrix protein). This indicates that such ghosts could be used to determine the conservation or dispersion of matrix protein during growth of the cell. Lugtenberg et al. (13) have shown that this matrix protein corresponds with their protein b when isolated from B strains of *E. coli* but consists of two proteins, b and c, when isolated from K-12 strains. B strains of *E. coli* do not contain protein c, and the original report of matrix protein was from a B-derived strain of *E. coli*.

This report describes results obtained when cells of *E. coli* K-12 and B/r were pulse-labeled with [³H]histidine and subsequently allowed to continue growing in the presence of low concentrations of penicillin to inhibit division (17). An experiment involving pulse-labeling of pre-formed filaments to determine any localization at the time of incorporation is also described.

MATERIALS AND METHODS

Strains and growth conditions. The strains used were *E. coli* KM7 K-12 Thr⁻ Leu⁻ (14) and *E. coli* B/r ATCC 12407 (9).

Cells were grown in minimal medium (M9 salts [1]) plus 0.2% glucose plus required amino acids at 20 µg/ml in a shaking water bath at 37°C. Doubling times were 60 min for KM7 and 41 min for B/r.

Penicillin was used at concentrations of 60 U/ml for KM7 and 30 U/ml for B/r.

Labeling with [³H]histidine. Radioactive histidine was chosen as the label because it is not inserted into the lipoprotein described by Braun and Rehn (6) which is covalently bound to the mucopeptide.

Growing cells were concentrated on a HAWP filter membrane (Millipore Corp.) (pore size, 0.45 µm) and resuspended in a small volume of growth medium containing [³H]histidine (47 Ci/mmol) at 20 µCi/ml (0.066 µg/ml) for the required pulse time. The pulse was stopped by washing the cells on a Millipore membrane filter with phosphate-buffered saline containing nonradioactive histidine at 100 µg/ml.

Ghost preparation. Washed cells were suspended in extraction buffer containing 2% sodium dodecyl sulfate in 10 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.3, containing 10% glycerol and 0.7 M 2-mercaptoethanol and heated to 60°C for 30 min. The resulting ghosts were washed three times with distilled water. Ghosts prepared by this technique were analyzed on polyacrylamide gels, and it was found, as other workers have found (13, 16), that matrix protein was the only protein present.

Sacculi controls were prepared by heating a portion of each cell sample to 100°C for 30 min.

Autoradiography. Ghosts were spread on clean glass microscope slides, dried, and overlaid with a thin layer of Ilford L-4 nuclear emulsion. Slides were kept in light-proof boxes and subsequently developed using Kodak D19b developer and Kodak F24 fixer. Autoradiograms were stained for 30 min with 1% methyl violet.

Collection of data. Autoradiograms were examined in a Zeiss Photo-Microscope, and photographs were taken by using Pan-F film (Ilford). Prints were made, and the positions of the grains along each filament were measured. Grain positions were ex-

pressed as fractional distances from the nearer of the two cell poles.

RESULTS

Figures 1 and 2 show the grain distribution obtained when KM7 cells were pulsed with [^3H]histidine for 10 min, chased with unlabeled histidine, and allowed to continue growing for two mass doublings in the presence of penicillin (60 U/ml). Autoradiograms of ghosts were prepared and grain positions were measured as described above. The grains appear to be randomly distributed over the entire surface of the ghosts.

Figure 3 shows the grain distribution obtained when B/r cells were pulsed with [^3H]histidine for 5 min and treated as above. Again the grains appear to be distributed at random over the surface of the ghosts.

Figure 4 shows the grain distribution given when filaments of KM7 cells, formed by treating growing cells with penicillin (60 U/ml) for two mass doublings, were pulsed with [^3H]histidine for 1 min immediately before ghost preparation. The grains are randomly located over the surface of the ghosts.

Figure 5 shows a control preparation of sacculi prepared from KM7 cells by heating Rosenbusch ghosts to 100°C for 30 min to remove matrix protein. The peptidoglycan sacculi can clearly be seen to be unlabeled.

Figure 6 shows a control preparation obtained by mixing equal proportions (mass) of penicillin-induced filaments labeled with [^3H]histidine and unlabeled normal-sized cells of *E. coli* KM7 before ghost preparation. The label is clearly confined to the filaments, indicating that matrix protein does not dissociate and reassociate during ghost preparation.

DISCUSSION

Matrix protein (b and c) represents approximately 35% of the total outer membrane protein

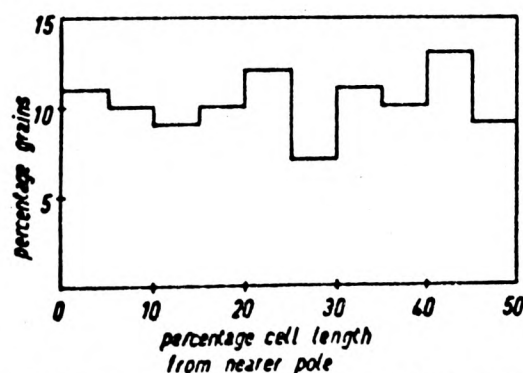


FIG. 1. Distribution of grains (304 grains measured) on the surface of cells ghosts of *E. coli* KM7. Cells were pulse-labeled with [^3H]histidine for 10 min and subsequently grown for two mass doublings in the presence of 60 U of penicillin per ml. The distance of each grain from the nearer pole was measured and expressed as a fraction of the total cell length.

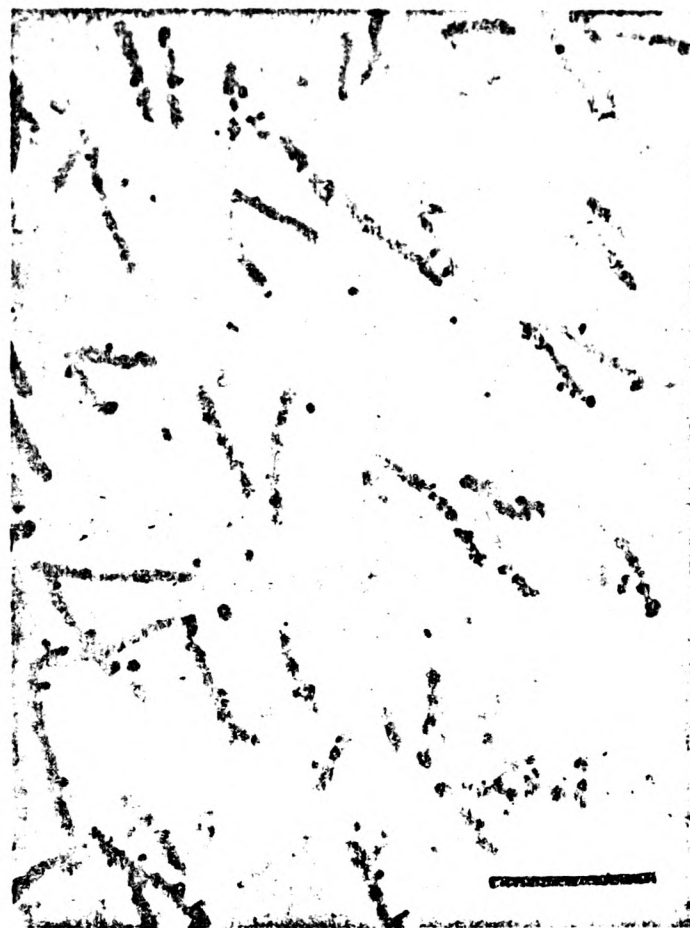


FIG. 2. Photomicrograph of ghosts described in Fig. 1. Bar is 10 μm .

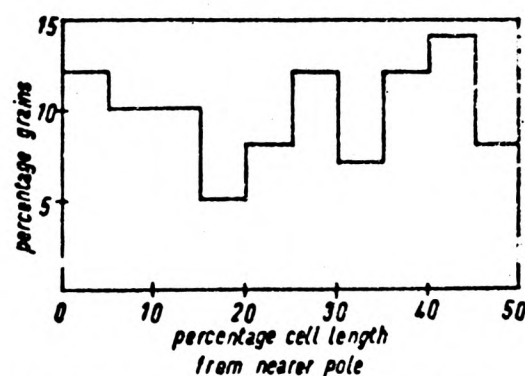


FIG. 3. Distribution of grains (102 grains measured) on the surface of cell ghosts of *E. coli* B/r. Cells were pulse-labeled with [^3H]histidine for 5 min and subsequently grown for two mass doublings in the presence of 30 U of penicillin per ml. Data expressed as in Fig. 1.

of the cell envelope in *E. coli* (13) and appears to be arranged in a lattice structure of hexagonal symmetry (16). It has been shown that matrix protein is closely associated with lipopolysaccharide, and it has been suggested that matrix protein, together with lipopolysaccharide, forms hydrophilic pores in the outer membrane (13).

The experiments described in this communication suggest that matrix protein is not spatially conserved in the cell envelope and tends to move freely and at random over the surface. In addition, it would appear from the experiments with preformed filaments that it is not even localized

for short periods at the time of incorporation. Although matrix protein does not appear to dissociate and reassociate during ghost preparation (Fig. 6) and has been shown to maintain a regular pattern when isolated in conjunction with mucopeptide (16), the possibility still exists that the random distribution of label observed in this communication is a result of procedural artifacts which cause relocation of matrix protein within individual cells, and unfortunately the technology to prove or disprove this does not as yet exist.

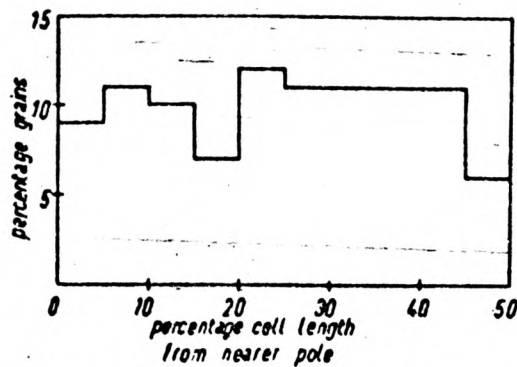


FIG. 4. Distribution of grains (364 grains measured) on the surface of cell ghosts of *E. coli* KM7. Cells were grown for two mass doublings in the presence of 60 U of penicillin per ml before being pulse-labeled with [3 H]histidine for 1 min immediately before ghost preparation.

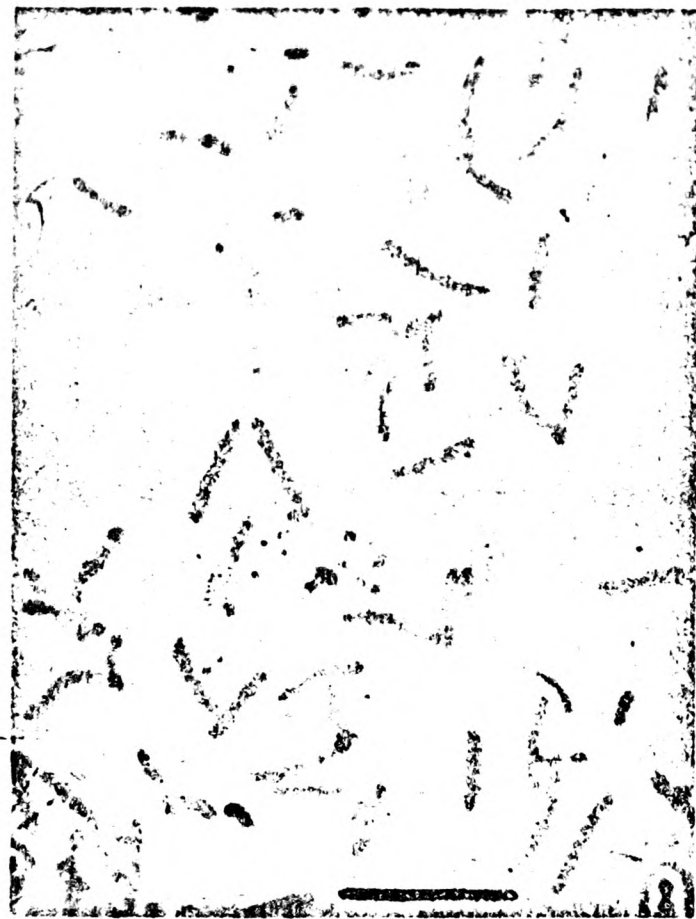


FIG. 5. Photomicrograph of sacculi prepared from [3 H]histidine-labeled ghosts of *E. coli* KM7 cells by heating to 100°C in sodium dodecyl sulfate to remove matrix protein. Bar is 10 μ m.

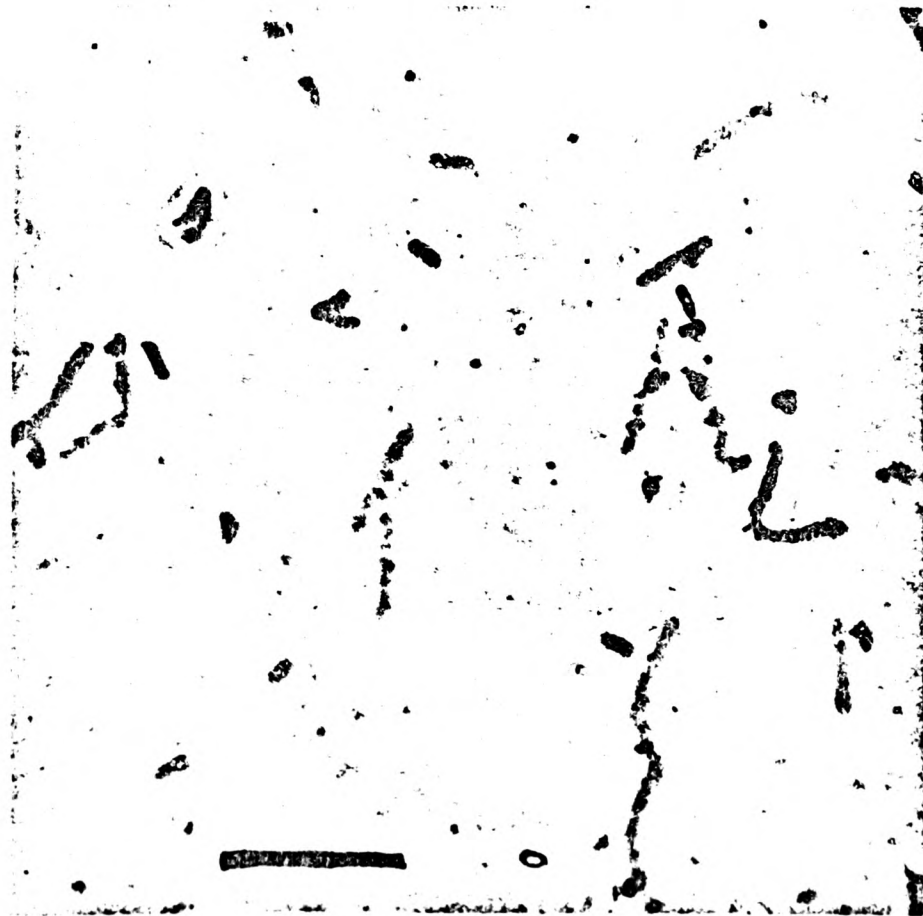


FIG. 6. Photomicrograph of ghosts prepared from a mixture of [3 H]histidine-labeled filaments and unlabeled normal-sized cells of *E. coli* KM7 mixed in equal proportions (mass) before ghost preparation and autoradiography.

It has been shown that mutant cells of *E. coli* which lack matrix protein can exist and grow quite normally without it (2, 10). This, combined with the information that the proportion of matrix protein and other outer membrane proteins can vary with cultural conditions (13), and the results shown in this paper, suggest that matrix protein is not part of any conserved structure in the cell envelope.

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LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages, 1st ed., p. 466. Interscience, New York.
2. Alphen, W., B. Lugtenberg, and W. Berendsen. 1976. Heptose deficient mutants of *Escherichia coli* K12 deficient in up to three major outer membrane proteins. *Mol. Gen. Genet.* 147:263-269.
3. Beachey, E. H., and R. M. Cole. 1966. Cell wall replication in *Escherichia coli* studied by immunofluorescent and immunoelectron microscopy. *J. Bacteriol.* 92:1245-1251.
4. Begg, K. J., and W. D. Donachie. 1977. Growth of the *Escherichia coli* cell surface. *J. Bacteriol.* 129:1524-1536.
5. Bisset, K. A., and P. E. Pease. 1957. The distribution of flagella in dividing bacteria. *J. Gen. Microbiol.* 16:382-384.
6. Braun, V., and K. Rehn. 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein lipoprotein) of the *E. coli* cell wall. *Eur. J. Biochem.* 10:426-438.
7. Donachie, W. D., and K. J. Begg. 1970. Growth of the bacterial cell. *Nature (London)* 227:1220-1224.
8. Green, E. W., and M. Schaechter. 1972. The mode of segregation of the bacterial cell membrane. *Proc. Natl. Acad. Sci. U.S.A.* 69:2312-2316.
9. Helmstetter, C. E. 1967. Rate of DNA synthesis during the division cycle of *E. coli* B/r. *J. Mol. Biol.* 24:417-428.
10. Henning, U., and I. Haller. 1975. Mutants of *Escherichia coli* K12 lacking all "major" proteins of the outer cell envelope membrane. *FEBS Lett.* 55:161-164.
11. Leal, J., and H. Marcovich. 1971. Segregation of phage receptors T6 during cell division in *Escherichia coli* K12. *Ann. Inst. Pasteur Paris* 120:467-474.
12. Lin, E. C. C., Y. Hirota, and F. Jacob. 1971. On the process of cellular division in *Escherichia coli*. *J. Bacteriol.* 108:375-385.
13. Lugtenberg, B., R. Peters, H. Bernheimer, and W. Berendsen. 1976. Influence of cultural conditions and mutations on the composition of the outer membrane proteins of *Escherichia coli*. *Mol. Gen. Genet.* 147:251-262.
14. Matzura, H., S. Molin, and O. Maaløe. 1971. Sequential biosynthesis of the β and β' subunits of the DNA dependent RNA polymerase from *Escherichia coli*. *J. Mol. Biol.* 59:17-25.
15. Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. *J. Biol. Chem.* 247:3962-3972.
16. Rosenbusch, J. P. 1974. Characterization of the major envelope protein from *Escherichia coli*. Regular arrangement on the peptidoglycan and unusual dodecyl sulphate binding. *J. Biol. Chem.* 249:8019-8029.
17. Starka, J. 1971. Formation et stabilite osmotique des formes filamenteuses d'*Escherichia coli* induites par la penicilline. *Ann. Inst. Pasteur Paris* 121:149-159.
18. Tsukagoshi, N., P. Fielding, and C. F. Fox. 1971. Membrane assembly in *Escherichia coli*. Segregation of preformed and newly formed membrane into daughter cells. *Biochem. Biophys. Res. Commun.* 44:497-502.